Altérations du muscle squelettique humain lors du vieillissement associé ou non au syndrome métabolique et identification de nouveaux marqueurs

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Résumé

Le vieillissement musculaire (sarcopénie) conduit inéluctablement à une perte d'autonomie, et à une moindre capacité à lutter contre les agressions métaboliques. Or, les mécanismes mis en jeu sont complexes et restent mal connus. Ainsi, au cours de cette thèse, une étude protéomique comparative a été développée afin d'identifier de nouveaux biomarqueurs potentiels de la sarcopénie chez la femme âgée post-ménopausée, et 73 protéines exprimées différentiellement dans le muscle âgé ont été identifiées.

En plus des altérations du muscle squelettique, l'âge est connu comme étant un facteur favorisant l'apparition du syndrome métabolique (SM), facteur de risque pour les maladies cardiovasculaires et le diabète de type II. Cependant, les effets du SM sur le muscle squelettique des personnes âgées sont peu décrits dans la littérature. Des marquages immunohistologiques ont été réalisés à partir de biopsies du muscle *vastus lateralis* provenant de personnes jeunes (25 ans) et âgées avec ou sans SM (75 ans), afin de décrire les altérations structurales et fonctionnelles du muscle squelettique liées à l'âge et au MS. Les résultats montrent une atrophie des fibres de type II ayant une déformation accrue lors du vieillissement. Chez les personnes âgées atteintes de SM, l'aire des fibres est augmentée par rapport aux personnes âgées contrôles, et une forte diminution de l'activité cytochrome c oxydase a été observée. De plus, le vieillissement et plus particulièrement le SM sont associés à une forte accumulation de lipides intramusculaires. Enfin, alors que peu de différences ont été observées chez les personnes âgées contrôles, le contenu en capillaire est fortement altéré chez les individus atteints de SM. Par la suite, une étude protéomique comparative a permis d'identifier 42 biomarqueurs potentiellement impliqués dans le vieillissement musculaire et/ou dans le syndrome métabolique.

L'ensemble des résultats obtenus au cours de cette thèse devrait permettre d'améliorer notre compréhension des facteurs impliqués dans le développement de la sarcopénie, et pourrait permettre d'identifier à la fois de nouvelles voies de régulation et suggérer des cibles thérapeutiques potentielles.

Mots clés : vieillissement, muscle squelettique humain, types de fibres musculaires, syndrome métabolique, biomarqueurs.

Abstract

Muscle aging (sarcopenia) contributes to both loss of autonomy and decreased capacity to prevent metabolic aggressions, but the mechanisms involved are complex and remain unclear. Therefore in this thesis, we have undertaken a top-down differential proteomic approach to reveal novel potential biomarkers of sarcopenia, and 73 differentially expressed proteins were identified.

In addition to alterations of skeletal muscle, aging favors metabolic syndrome (MS), a risk factor for cardiovascular disease and type II diabetes. However, the effects of MS on skeletal muscle in old individuals have poorly been investigated. Immunohistochemical studies were performed with *vastus lateralis* muscle biopsies from young (25 years) and old (75 years) men with and without MS, to reveal the importance of age-dependent and MS-associated modifications on fiber-type characteristics. An atrophy of type-II fibers and altered fiber shape characterized muscle aging in lean healthy men. In contrast, increased cross sectional area of fibers, and reduced cytochrome c oxidase activity in all fiber types characterized MS, even in active elderly men. Moreover, aging and particularly MS were associated with accumulation of intramyocellular lipid droplets. Finally, while few differences were observed in lean healthy men, the capillary supply was strongly altered in old men with MS. Thereafter, a differential proteomic approach identified 42 potential biomarkers implicated in muscle aging and/or in metabolic syndrome.

Overall the results obtained in this thesis may improve our understanding of the factors influencing sarcopenia, and may both identify new regulatory pathways and provide potential therapeutical targets.

Keywords: aging, human skeletal muscle, fiber types, metabolic syndrome, biomarkers.

A mes parents...

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Liste des abréviations

2DGE: Two-Dimensional gel electrophoresis
AACE: American Association of Clinical Endocrinologists
ACADS: short-chain specific acyl-CoA dehydrogenase
ACTH: Adrénocorticotrophine
ADNmt: ADN mitochondriale
ADP: Adénosine DiPhosphate
ADRP: Adipose Differentiation-Related Protein ou Adipophiline
AG: Acide gras
AGE: Advanced Glycosylation and products
AHA: American Heart Association
ALDH2: Aldéhyde déshydrogénase
ANKRD2: Ankyrin repeat domain-containing protein 2
APA : Allocation Personnalisée d'Autonomie
AR : Arthrite Rhumatoïde
ARNm: Acide RiboNucléique messager
ATGL: Adipose Triglyceride Lipase
ATP: Adénosine TriPhosphate
C/F: Capillary-to-fiber number
Ca^{2+}: Calcium
CCT2: Chaperonin Containing T-complex polypeptide 1 subunit 2
CD: Capillary Density
CFPE: Capillary-to-fiber Perimeter Exchange
COX: Cytochrome c Oxydase
CRM: Caloric Restriction Mimetics
DGC: Dystroglycane
DHEA: Déhydroépiandrostérone
DHPR: Dihydropyridine Receptor
DRESS : Direction de la Recherche, des Etudes, de l'évaluation et des Statistiques
E_2: Oestradiol cyclique
ECH1: delta(3,5)-delta(2,4)-dienoyl-CoA isomerase
EGIR: European Group for study of Insulin Resistance
ELC: Essential Light Chain
FABP3: Fatty Acid Binding Protein 3
FABP4: Fatty Acid Binding Protein 4
FABPc: Fatty Acid Binding Protein cytosolic
FABPpm: plasma membrane-associated Fatty Acid Binding Protein
FADH: Flavin Adenine Dinucleotide
FAT: Fatty Acid Transporter
FGF: Fibroblast Growth Factor
FHL3: Four and Half LIM domain protein 3
FSH: Follicule-Stimulating Hormone
GABA: acide γ-aminobutyrique
GH: Growth Hormone
GLOD4: Glyoxylase domain-containing protein 4
GLUT4: Glucose Transporter type 4

GOT1: aspartate aminotransférase cytosolique
H_2O_2: Peroxyde d'hydrogène
HGF: Hepatocyte Growth Factor
HSL: Hormone Sensitive Lipase
HSP: Heat Shock Protein
IDF: International Diabetes Federation
IFNγ: Interféron gamma
IGF-I: Insulin-like Growth Factor I
IL : Interleukine
INSEE : Institut National de la Statistique et des Etudes Economiques
JIS: Joint Interim Statement
LC/PF: Length of capillary/Perimeter Fiber
LD: Lipid Droplet
LH: Luteinizing Hormone
MEC : Matrice Extracellulaire
MGL: MonoGlyceride Lipase
MHC: Myosin Heavy Chain
MLC: Myosin Light Chain
MLP: Muscle LIM Protein
MRF: Muscle Regulatory Factor
MyBP: Myosin Binding Proteins
MYL6B: Myosin Light Chain 6B
NADH: Nicotinamide Adenine Dinucleotide
NCEP ATP II: National Cholesterol Education Program Adult Treatment Panel III
NHLBI: National Heart Lung and Blood Institute
NK: Natural killer
NO: Nitric oxide
$O_2^{\cdot -}$: anion péroxyde
OA: Ostéoarthrite
OCDE : Organisation de Coopération et de Développement Economique
PAT: Périlipine/ADRP/TIP47
PCR: Polymerase Chain Reaction
PG: Protéoglycane
PGC-1α: Peroxisome proliferator-activated receptor Gamma Coactivator alpha
pI: point Isoélectrique
PIB : Produit Intérieur Brut
PM: Poids Moléculaire
RE: Réticulum Endoplasmique
RLC: Regulatory Light Chain
RMN: Résonnance Magnétique Nucléaire
ROS : Reactive Oxygen Species
RS : Réticulum Sarcoplasmique
RyR: Ryanodine Receptor
SDH : Succinate Déshydrogénase
SERCA: Sarcoplasmique/Endoplasmique Réticulum Calcium-ATPase
SF: Shape Factor
SM : Syndrome Métabolique
TAV: Tissu Adipeux Viscéral
TG: Triglycéride
TGF-β: Transforming Growth Factor beta

TIP47: Tail-Interacting Protein of 47 kDa
TNFα: Tumor Necrosis Factor alpha
TNNT1: slow troponin T
TNNT3: fast troponin T
UPS: Ubiquitin Proteasome System
VEGF: Vascular Endothelium Growth Factor
VO2max: consummation maximale d'oxygène
WHO: World Health Organization

Introduction générale

Le vieillissement de la population est un phénomène mondial et inévitable. La proportion des personnes âgées ne cesse d'augmenter : elle est passée de 8 % en 1950 à 11 % en 2009 et, selon les prévisions, devrait atteindre 22 % d'ici 2050. Ceci pose de graves problèmes concernant à la fois la santé publique et l'économie.

Le vieillissement est caractérisé par un déclin de nombreuses fonctions physiologiques et par des modifications importantes de la composition corporelle, dont la perte de masse musculaire est probablement une des conséquences majeures. A partir de 50 ans, on estime que l'Homme subit une perte d'environ 1 à 2 % de masse musculaire par an, menant à une réduction d'environ 50 % de la masse musculaire chez les personnes âgées de plus de 80 ans. Cette atrophie musculaire liée au vieillissement est également appelée « sarcopénie » et se traduit par une augmentation du risque de chutes, une progressive incapacité à effectuer des activités basiques de la vie quotidienne, une perte d'autonomie, et conduit finalement à une réduction de l'espérance de vie.

Le muscle squelettique représente 40 % des protéines totales de l'organisme. En dehors de son rôle fonctionnel, le muscle possède un autre rôle majeur puisqu'il est un réservoir important d'acides aminés mobilisables par l'organisme en cas d'agressions d'ordre nutritionnel, infectieux ou traumatique. L'atrophie musculaire chez la personne âgée est donc responsable à la fois d'une réduction de la mobilité, mais aussi d'une moindre capacité de réponse aux agressions et ainsi d'une augmentation de nombreuses pathologies. Bien que la sarcopénie semble prévalente chez les personnes âgées, le manque d'une définition claire et précise rend son diagnostic difficile. Ainsi, les conséquences et les mécanismes moléculaires impliqués lors du vieillissement musculaire restent actuellement très mal compris.

Par ailleurs, le vieillissement est également associé à l'apparition de nombreuses pathologies, telles que le diabète de type II ou les maladies cardiovasculaires. Ces dernières sont un fléau mondial qui augmente considérablement le taux de mortalité chez les personnes âgées. En 1988, Reaven *et al.* ont décrit un ensemble de facteurs de risque favorisant l'apparition de ces pathologies : le syndrome métabolique. La constatation la plus flagrante est que la prévalence de ce syndrome est fortement dépendante de l'âge. Ce phénomène est très clair en France où la prévalence passe de moins de 5 % chez les individus âgés de 30 à 39

ans à 30 % chez les personnes âgées de 60 à 69 ans. Cependant, malgré la perte de masse et/ou de force musculaire observée chez les personnes âgées atteintes de syndrome métabolique, les altérations du muscle squelettique chez ces individus sont peu connues.

Il devient donc indispensable de mieux comprendre les mécanismes cellulaires et moléculaires impliqués lors du vieillissement musculaire, afin de prévenir cette perte de muscle squelettique et, à terme, de développer de nouvelles stratégies thérapeutiques ou nutritionnelles. Par ailleurs, la prévention et une meilleure connaissance des altérations musculaires associées à l'apparition de certaines pathologies, comme le syndrome métabolique, joueront un rôle crucial pour « mieux vieillir » et pour améliorer ainsi la santé et la qualité de vie des personnes âgées.

Etude bibliographique

Chapitre I : Le vieillissement chez l'Homme

I. Le vieillissement de la population

1. Aspects démographiques

L'actuel vieillissement de la population est un phénomène mondial sans précédent, qui est à la fois inévitable et prévisible. On parle de vieillissement de la population lorsque l'augmentation du nombre de personnes âgées (c'est-à-dire les personnes de 60 ans ou plus) s'accompagne d'une diminution du nombre d'enfants (les personnes de moins de 15 ans) et ainsi qu'une diminution de la proportion de personnes d'âge actif (entre 15 et 59 ans).

Le vieillissement de la population est omniprésent puisqu'il concerne presque tous les pays et il s'explique principalement par une baisse de la fécondité associée à un allongement de l'espérance de vie (Figure 1). Au niveau mondial, le nombre de personnes âgées augmente de 2,6% par an, soit nettement plus rapidement que la population dans sa globalité, qui croît à un taux de 1,2% (Economic & Division, 2010). Depuis 1950, la proportion de personnes âgées augmente de manière constante : elle est passée de 8% en 1950 à 11% en 2009 et, selon les prévisions, devrait atteindre 22% en 2050 (Dupâquier, 2006).

Cette augmentation de la longévité pose plus particulièrement la question du financement des retraites dans les futures décennies. En effet, le rapport de soutien potentiel, c'est-à-dire le nombre de travailleurs potentiels pour une personne âgée, a tendance à baisser à mesure que la population vieillit: il est passé de 12 à 9 entre 1950 et 2009, et devrait tomber à 4 en 2050 (Economic & Division, 2010). Ainsi, cette diminution a des conséquences importantes sur les mécanismes de protection sociale, notamment sur les régimes de retraite par répartition dans lesquels ce sont les actifs qui financent les pensions des retraites par leurs cotisations sociales.

Cependant, si le vieillissement de la population est un phénomène mondial, chaque pays se trouve à un stade différent de la transition. La figure 2 montre comment la proportion des personnes âgées augmente dans le monde. Dans les régions développées, les personnes âgées de 60 ans ou plus représentent actuellement plus d'un cinquième de la population et devraient en représenter près d'un tiers en 2050. Ainsi, selon l'INSEE, la France comptera 69 habitants âgés de 60 ans ou plus pour 100 habitants de 20 à 59 ans, soit deux fois plus qu'en

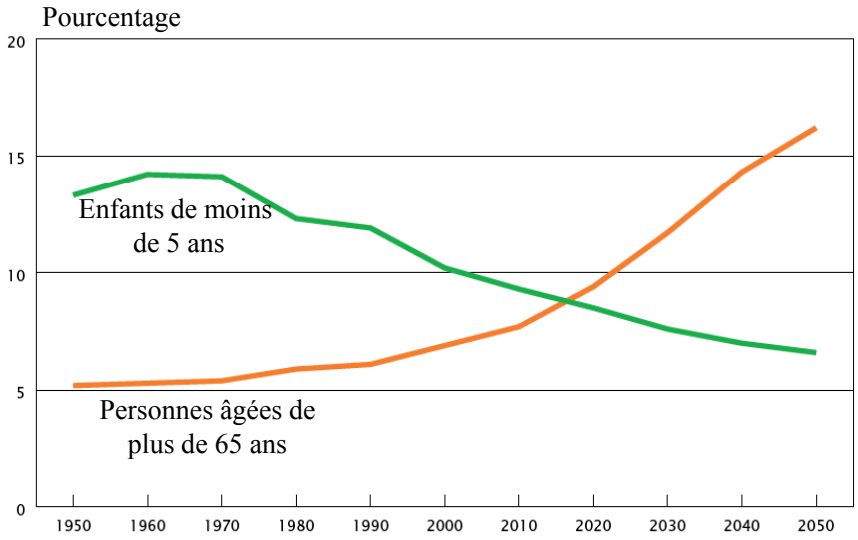

Figure 1: Pourcentage des enfants de moins de 5 ans et des personnes âgées de plus de 65 ans dans la population mondiale: 1950 à 2050.
D'après **Kinsella** et al, 2009

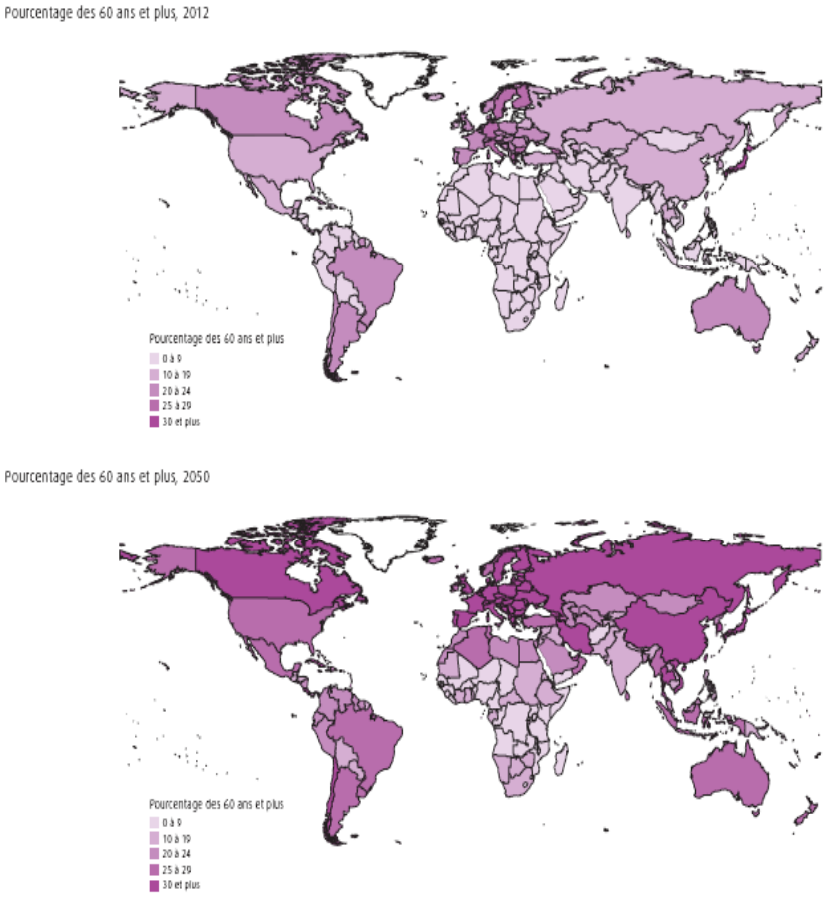

Figure 2: Pourcentage des personnes âgées 60 ans et plus dans le monde.
D'après « Une bonne santé pour mieux vieillir », Dossier pour la journée mondiale de la santé. Organisation Mondiale de la Santé, 2012

2005 (Robert-Bobée, 2006). Dans les pays en développement, les personnes âgées représentent aujourd'hui 8% de la population mais en 2050, elles devraient en représenter un cinquième, ce qui signifie que vers le milieu du siècle, ces pays devraient être au même stade que les pays développés aujourd'hui (Kinsella et al, 2009).

2. Enjeux économiques : dépenses de santé et dépendance

Depuis le milieu du XXe siècle, les économies des pays développés connaissent une augmentation continuelle de la part du produit intérieur brut (PIB) consacré aux dépenses de santé (Tableau 1). En France, la part des dépenses de santé dans le PIB est passée de 3,8% en 1960 à 11,6% en 2011 (Le Garrec et al, 2012).

L'idée que le vieillissement pourrait être à l'origine de cette augmentation est très répandue et discutée. Selon les prévisions de l'Organisation de Coopération et Développement Economique (OCDE), la part des dépenses de santé dans le PIB devrait passer de 7,7% en 2005 à 12,8% en 2050 (Dormont et al, 2006). Sur cette progression de 5,1 points de PIB, seulement 0,7 point serait attribuable aux changements démographiques, conférant au vieillissement un rôle mineur. Comme le montre la figure 3, la dépense individuelle de santé en France s'accroit de 50% pour la plupart des âges depuis 1992, et cette dérive peut être attribuée aux innovations médicales et aux changements des comportements des patients et des praticiens (Dormont & Hubert, 2012). La part due aux 65 ans et plus passe de 14,5% à 16,1% dans la même période, soit 1,6 point d'augmentation seulement.

En fait, en matière de dépenses de santé, il faut distinguer le vieillissement, c'est-à-dire l'augmentation de l'âge nominal, de la progression de la morbidité. Les progrès médicaux permettent de différer l'apparition des maladies à des âges plus tardifs, c'est donc la morbidité et non l'âge en soi qui explique le profil des dépenses de santé (Yang et al, 2003). En effet, la proximité de la mort a un impact considérable sur le niveau des dépenses (Figure 4). Il n'y a pas de doute que la forme croissante des dépenses par âge est en partie due au coût élevé de la prise en charge des personnes en fin de vie.

Outre la morbidité, la dépendance des personnes croît considérablement avec l'âge. La prise en charge de la dépendance, aussi appelée « soins de long terme », est un sujet très diffèrent de celui des dépenses de santé. Depuis plusieurs années, la perte d'autonomie liée au vieillissement est devenue une source de préoccupation pour les gouvernements mais aussi

Pays	1960	2010	Variation
France	3,8	11,6	+ 7,8
Allemagne *	6	11,6	+ 5,6
Royaume-Uni	3,9	9,6	+ 5.7
Suisse	4,9	11,2	+ 6.3
Etats-Unis	5,1	17,6	+ 12.5
Japon **	3	9,5	+ 6.5

Tableau 1: Pourcentage des dépenses totales de santé dans le PIB, évolution de 1960 à 2010 (* Allemagne: 1970-2010, ** Japon: 1960-2009).
D'après : Eco-santé OECD, 2012; www.stats.oecd.org

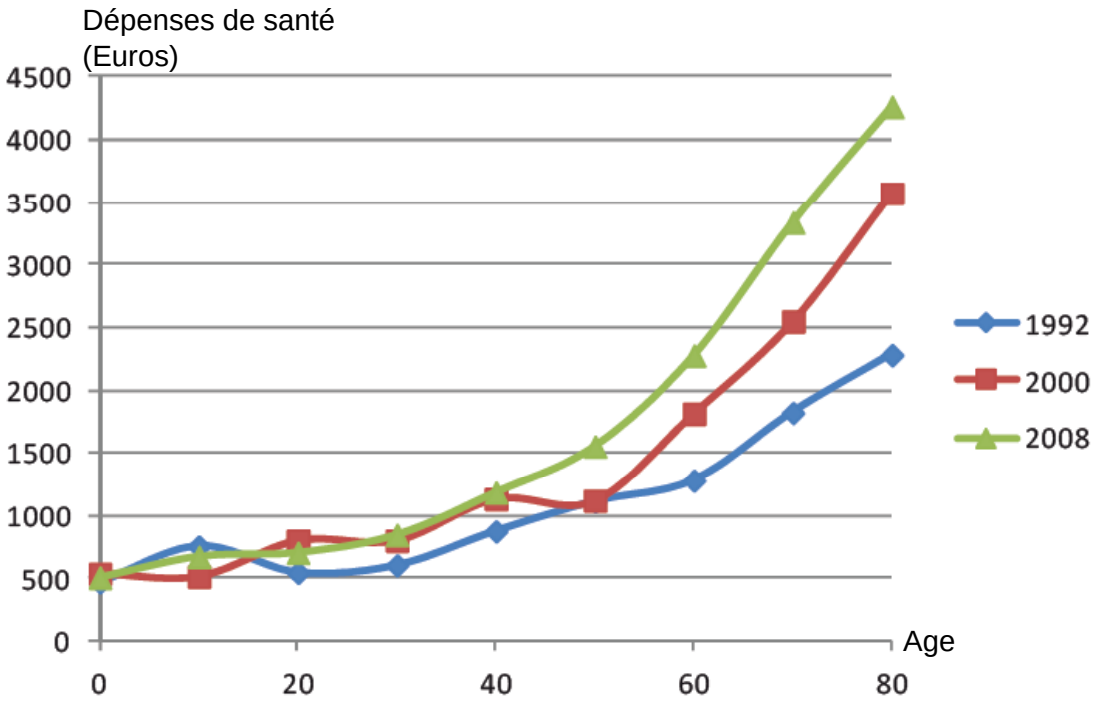

Figure 3: Dépenses de santé annuelle par tranche d'âge en 1992, 2000 et 2008.
D'après : **Dormont & Hubert,** 2012

pour l'ensemble des Français. Avec l'allongement de l'espérance de vie, de plus en plus de ménages y sont confrontés, et le financement de la perte d'autonomie liée au vieillissement est devenu un enjeu majeur. Les perspectives d'évolution du coût de la dépendance, à moyen et long terme, sont en grande partie déterminées par l'évolution du nombre de personnes âgées dépendantes, qui représente un réel enjeu démographique qu'on a pu parfois dénommer le « papy-boom ». D'après les projections réalisées par la DREES et l'INSEE, à partir des données relatives à l'APA, le nombre de personnes âgées dépendantes en France métropolitaine serait multiplié par 1,4 entre 2010 et 2030 et par 2 entre 2010 et 2060 (Figure 5) (Blanpin & Chardon, 2010). Le besoin de financement de la dépendance va donc mécaniquement augmenter de façon importante dans les années à venir, les estimations s'établissant autour de 10 milliards d'euros supplémentaires par an jusqu'à 2040.

Pour conclure, outre celui de trouver des réponses financières pérennes, l'un des défis posé est donc aussi d'améliorer la santé et la qualité de vie des personnes âgées. L'un des objectifs est de diminuer plusieurs facteurs de risque tels que le tabagisme, le diabète, l'hypertension artérielle, le cancer, etc. De même, la prévention et l'amélioration de physiopathologies déterminant l'état de dépendance, telle que la sarcopénie, joueront un rôle crucial pour mieux vieillir.

II. Effets du vieillissement normal sur l'organisme

1. Le système nerveux central

En ce qui concerne le système nerveux, il faut distinguer le vieillissement normal, c'est-à-dire sans pathologie bien définie, du vieillissement pathologique. Alors que les pathologies neurodégénératives liées à l'âge comme la maladie d'Alzheimer ou de Parkinson sont largement associées à une perte massive de neurones corticaux, l'idée d'une perte neuronale lors du vieillissement normal semble être erronée. En effet, les modifications neurophysiologiques et neurobiologiques du système nerveux central pourraient résulter principalement d'une perte de volume des neurones et de leurs dentrites, induisant une altération de la plasticité neuronale, et non d'une mort des neurones (Morrison & Hof, 1997). Aussi, le vieillissement normal est associé à une raréfaction de la substance blanche (Sullivan

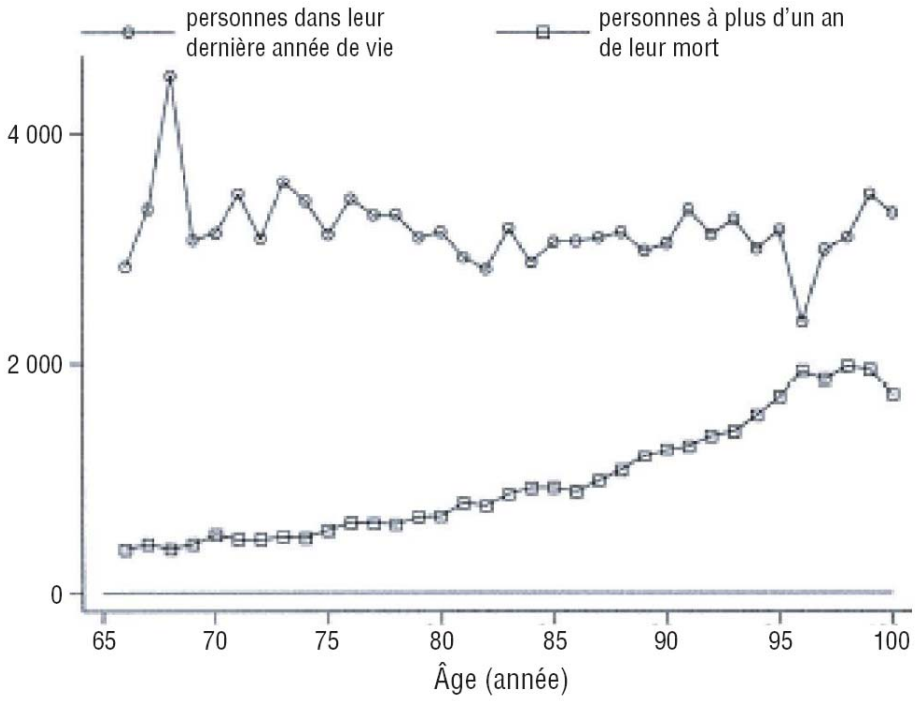

Figure 4: Dépenses mensuelles de santé ($ base 1998) en fonction de l'âge, en distinguant ceux qui décèdent dans l'année (courbe du haut) et ceux qui ne décèdent pas dans l'année (courbe du bas).
D'après :**Yang** et al, 2003

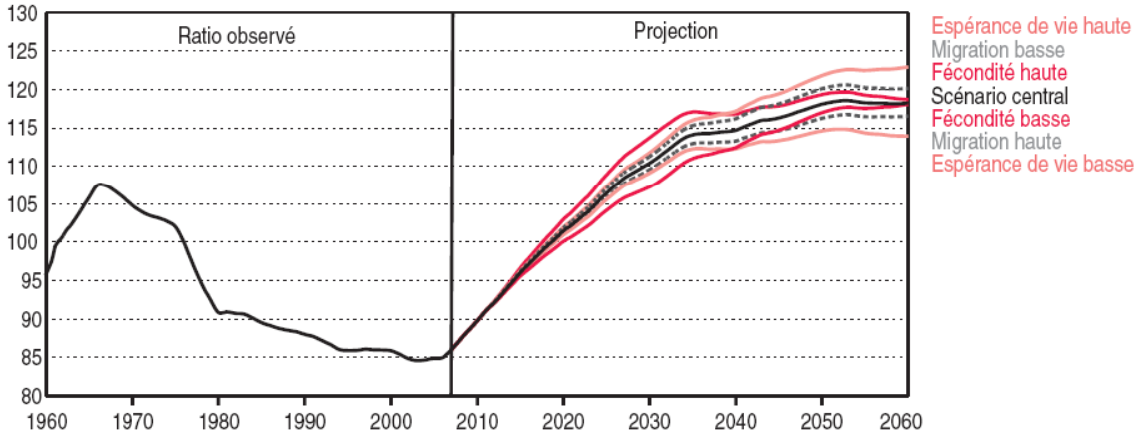

Figure 5: Evolution du ratio de dépendance économique entre le nombre de personnes d'âge « inactif » (c'est-à-dire de moins de 20 ans ou de plus de 60 ans) pour 100 personnes d'âge « actif » (de 20 à 59 ans).
D'après : **Blanpin & Chardon**, 2010

& Pfefferbaum, 2006) et à une diminution de certains neurotransmetteurs comme la dopamine ou l'acétylcholine (Mora et al, 2012). Comme le montre la figure 6, ces molécules sont impliquées dans de nombreuses fonctions et une perturbation de leur sécrétion durant le vieillissement va avoir différentes conséquences :

- augmentation des temps de réaction et réduction modérée des performances mnésiques concernant l'acquisition de nouvelles informations (Dykiert et al, 2012; Hultsch et al, 2002).
- réduction et déstructuration du sommeil dues à une désorganisation des rythmes circadiens (Pace-Schott & Spencer, 2011).
- diminution de la sensation de soif (Bossingham et al, 2005).

Le vieillissement est également associé à des changements au niveau du système nerveux autonome, affectant la régulation des fonctions de nombreux organes comme le cœur. Malgré une hyperactivité sympathique observée chez les personnes âgées (Ebert et al, 1992), ces changements conduisent à une réduction de la réponse aux stimuli environnementaux ou intrinsèques en raison d'une diminution de sensibilité des récepteurs aux catécholamines (Hotta & Uchida, 2010).

2. Le système cardiovasculaire

Il a été établi qu'une série de changements structuraux et fonctionnels marquait le vieillissement du muscle cardiaque et du réseau vasculaire (Priebe, 2000).

Avec l'âge, le poids du cœur augmente et la paroi ventriculaire s'épaissit (Lakatta, 2003). Le nombre de myocytes s'amenuise, mais leur taille augmente (Olivetti et al, 1995). La matrice de collagène se développe, induisant une fibrose pariétale et un épaississement de la paroi du ventricule gauche, ce qui rigidifie la chambre ventriculaire. Ainsi, les temps de contraction/relaxation s'allongent, ce qui influe sur la vitesse de remplissage ventriculaire. Cette altération de la fonction diastolique est habituellement compensée par une augmentation de la pression auriculaire (Pearson et al, 1991). Le tissu nodal n'échappe pas au processus dégénératif. En effet, avec l'âge, il apparait fréquemment un dysfonctionnement sinusal résultant d'un appauvrissement en cellules sinusales (Shioi & Inuzuka, 2012).

Le vieillissement de la paroi artérielle se caractérise par une augmentation du calibre artériel et par un épaississement des parois lié à la prolifération du collagène, augmentant ainsi la tension artérielle systolique (Lakatta, 1990). A l'inverse, l'élastine subit une dégradation entraînant une diminution de la capacité de dilatation des gros vaisseaux. De plus, les cellules musculaires lisses prolifèrent et migrent dans l'espace sous-intima, et ceci est

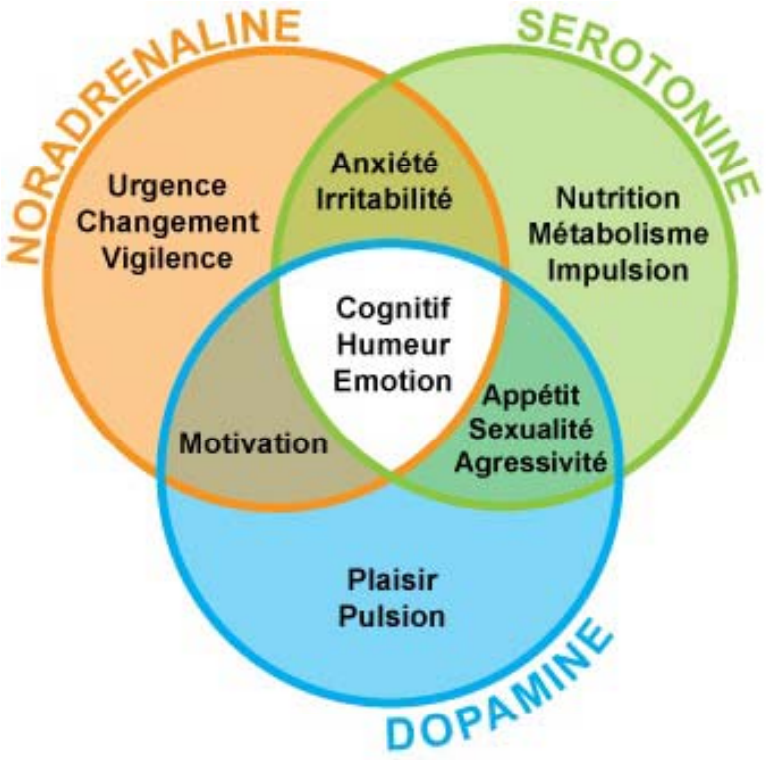

Figure 6: Effets des 3 neurotransmetteurs majeurs et états mentaux induits par leurs interactions.
D'après: **Stuart Ira F.** (2009) Human Physiology. *New York, NY: McGraw-Hill*.

accompagné de dépôts lipidiques et de macrophages, ce qui peut avoir des conséquences graves comme la formation de caillots sanguins (Wang & Bennett, 2012).

Ainsi, les altérations combinées du muscle cardiaque et du réseau vasculaire vont conduire à une augmentation importante de risque de maladies cardiovasculaires chez la personne âgée (Figure 7).

3. Le système immunitaire

Le vieillissement du système immunitaire, appelé immunosénescence, est partiellement responsable de l'augmentation de la prévalence et de la sévérité des maladies infectieuses, ainsi que de la faible efficacité de la vaccination chez la personne âgée (Weiskopf et al, 2009). L'immunosénescence touche différents types cellulaires impliqués aussi bien dans la réponse innée que dans la réponse adaptative. De plus, le vieillissement est associé à une diminution du renouvellement des cellules souches hématopoïétiques, cellules impliquées dans le renouvellement des cellules du système immunitaire (Lansdorp et al, 1994).

Parmi les cellules du système immunitaire adaptatif, la réponse médiée par les lymphocytes T et B est diminuée avec l'âge. En effet, le nombre de cellules T naïves chute chez la personne âgée, et ces cellules présentent de nombreuses anomalies comme un raccourcissement des télomères, une production réduite d'IL-2 ou encore une altération de leur expansion et de leur différenciation en cellules effectrices (Pfister et al, 2006). Ainsi leur capacité à médier une réponse immune contre de nouveaux antigènes est diminuée. Le nombre de cellules B, lui, reste inchangé avec l'âge, cependant leur stimulation par les cellules dendritiques est 70% moins efficace que chez une personne jeune (Aydar et al, 2002). Les cellules B mémoires présentent une susceptibilité réduite à l'apoptose et s'accumulent donc lors du vieillissement, conduisant à des expansions clonales de certaines spécificités des cellules B. Ces expansions peuvent ainsi limiter la diversité du répertoire et influencer le devenir de la vaccination chez le sujet âgé (Weksler & Szabo, 2000).

Avec l'âge, les cellules phagocytaires et les cellules Natural Killer (NK), cellules du système immunitaire inné, vont produire des taux plasmatiques élevés d'IL-6, IL-1β, et de TNFα (Bruunsgaard et al, 2003 ; Ershler & Keller, 2000 ; O'Mahony et al, 1998). Ces facteurs sont responsables d'une activation continue du système immunitaire chez la personne âgée, conduisant à une inflammation chronique appelée « inflamm-aging » (Franceschi et al, 2000). De plus, l'activité phagocytaire des neutrophiles et macrophages est diminuée (Lloberas &

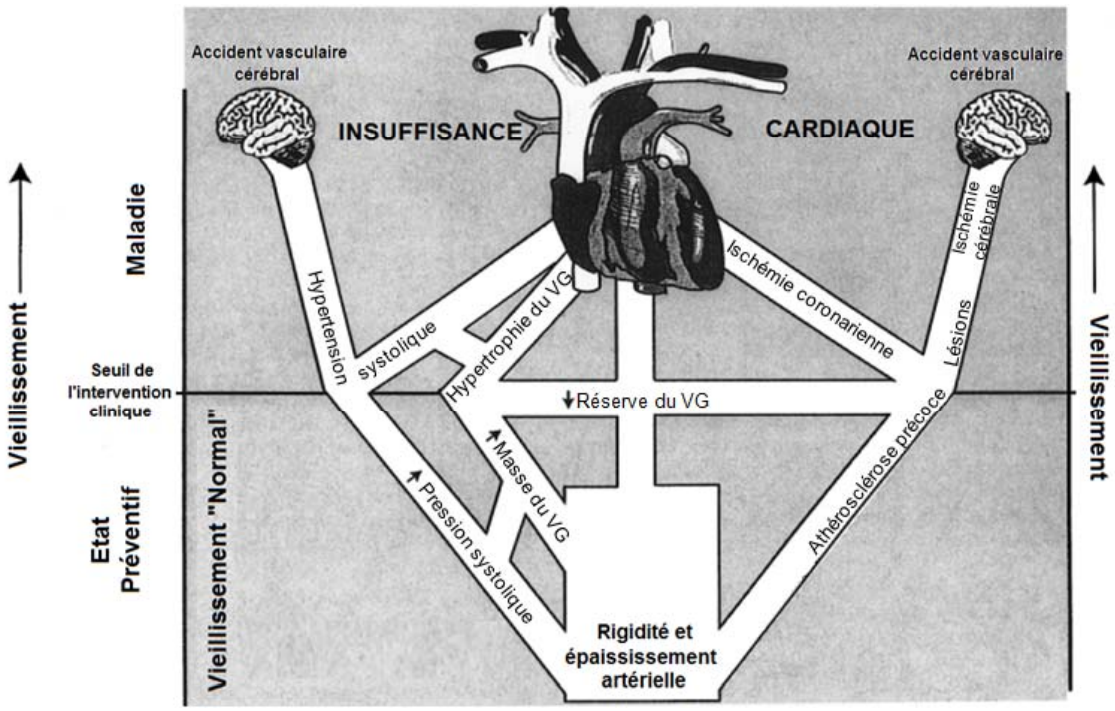

Figure 7: Changements cardiaques et vasculaires liés à l'âge, conduisant à des maladies cardiovasculaires lors du vieillissement. La ligne séparant la partie supérieure de la partie inférieure du schéma représente le point où le vieillissement « normal » (en-dessous de la ligne) conduira à des symptômes pathologiques (au-dessus de la ligne). VG: Ventricule gauche.
D'après: **Priebe**, 2000

Celada, 2002; Wenisch et al, 2000), ainsi que la présentation des antigènes aux lymphocytes T par les cellules dendritiques (Agrawal et al, 2007). Enfin, bien que le nombre de cellules NK semble augmenté avec l'âge, leur toxicité et leur production d'IFNγ est réduite (Borrego et al, 1999).

4. Le système endocrinien

Une perte de masse et de force musculaire, de densité osseuse ainsi qu'une augmentation de la masse grasse sont des conséquences du vieillissement. Ces altérations de la composition corporelle peuvent être liées au système endocrinien. Trois systèmes hormonaux montrent des taux d'hormones circulantes diminués lors du vieillissement et conduisent à la ménopause chez la femme et l'andropause chez l'homme, l'adrénopause et la somatopause (Epelbaum, 2008) (Figure 8).

La somatopause résulte d'une baisse de la sécrétion de l'hormone de croissance (GH) par l'hypophyse, causant une diminution de la production du facteur de croissance IGF-I (Insulin-like Growth Factor I) par le foie et d'autres organes (Ghigo et al, 1996). Les propriétés anaboliques de la GH ont suggéré que la manipulation de l'axe GH/IGF-I serait une option thérapeutique envisageable pour contrecarrer certains changements néfastes se produisant lors du vieillissement. Ainsi de nombreuses thérapies basées soit sur une administration de GH (Rudman et al, 1990), soit sur une stimulation de l'axe GH/IGF-I (Broglio et al, 2002; Corpas et al, 1992) ont été développées. Bien que ses traitements aient montré une amélioration de certaines fonctions comme une augmentation de la masse musculaire et une diminution de la masse grasse, les effets sont assez controversés et semblent être à l'origine de rétentions d'eau, d'intolérance au glucose ou encore de gynécomastie chez l'homme (Giordano et al, 2008). De plus, un niveau élevé d'IGF-I semble corrélé à un risque accru de développer un cancer, et notamment un cancer mammaire ou prostatique (Cohen et al, 2000).

En plus de la somatopause, la ménopause est un processus induisant des changements majeurs chez la femme âgée. La production d'œstradiol cyclique (E_2) durant les années de reproduction est remplacée par une production très basse et constante de cette hormone. Les causes de cette ménopause sont à la fois dues à un épuisement de la réserve de follicules ovariens mais également à une diminution de la sécrétion de FSH (Follicule-Stimulating Hormone) et de LH (Luteinizing Hormone) par le système hypothamalo-hypophysaire (Wise, 1999). Des traitements hormonaux substitutifs de la ménopause ont montré de nombreux

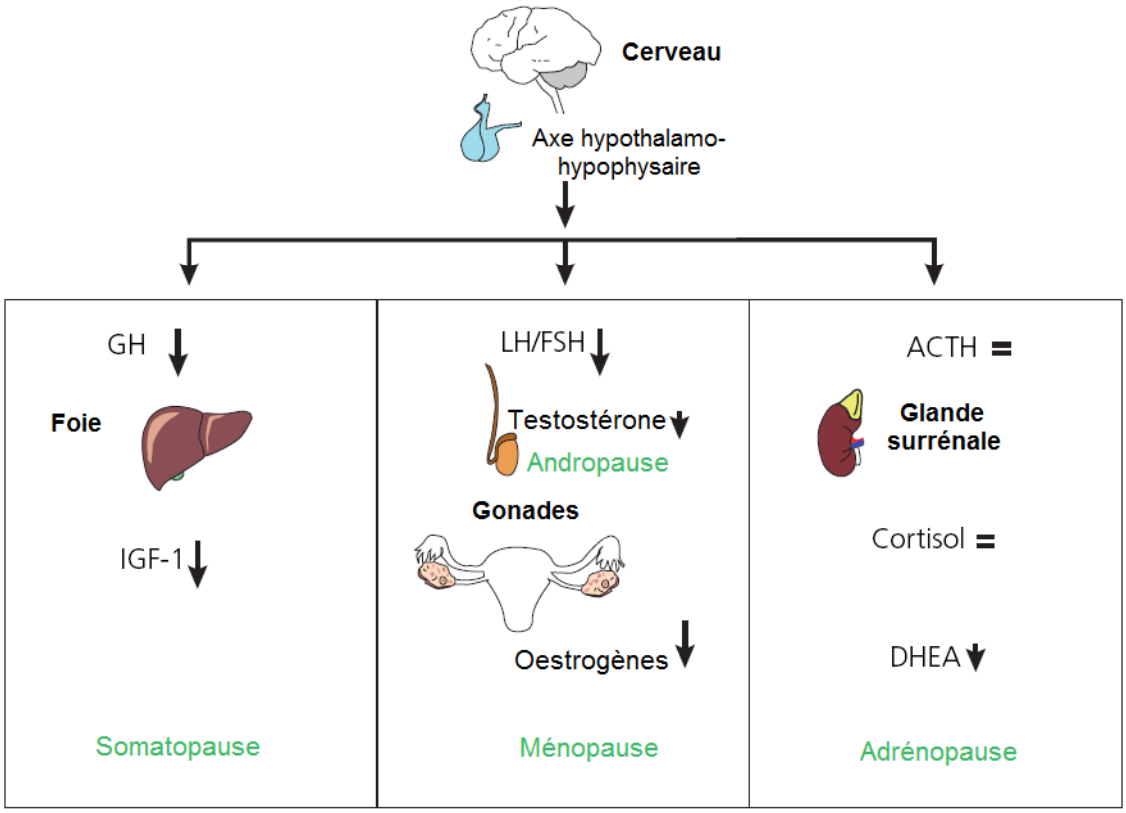

Figure 8: Principaux changements du système endocrinien lors du vieillissement, somatopause, ménopause/andropause et adrénopause. GH, Growth hormone; IGF-I Insulin-like growth factor I; LH, Luteinizing hormone; FSH, Follicule-stimulating hormone; ACTH, Adrénocorticotrophine; DHEA, Déhydroépiandrostérone.
D'après: **Epelbaum** et al, 2008

avantages quant à la prévention d'une perte de densité osseuse, de fonctions cognitives ou encore quant à l'apparition de l'athérosclérose (Grady et al, 1992). Cependant, ces traitements ont également montré des effets néfastes comme une augmentation de l'incidence du cancer mammaire (Fournier et al, 2009). Chez l'homme, les changements d'activité de l'axe hypothalamo-hypophyso-gonadique induisent à une baisse des taux de testostérone sérique, appelée andropause (Delev et al, 2009). Cependant, à l'inverse de la ménopause, ces changements sont progressifs, aléatoires et présentent une grande variabilité entre individus, rendant le diagnostic compliqué. Des traitements de supplémentation par testostérone sont prescrits et ont montré des effets bénéfiques comme une augmentation de la force musculaire ou de la fonction sexuelle. Cependant, le diagnostic et la dose de testostérone administrée en fonction de la personne doivent être optimisés afin d'éliminer les contre-indications existantes (Brawer, 2004).

De plus, lors du vieillissement, une baisse des niveaux circulants de déhydroépiandrostérone (DHEA) conduit à l'adrénopause. La DHEA est un précurseur de la testostérone et des œstrogènes, elle est sécrétée par la zone réticulaire de la glande surrénale en réponse à l'adrénocorticotrophine (ACTH). Etant donné que le niveau d'ACTH reste identique lors du vieillissement, le déclin de DHEA dans les deux sexes semble être causé par une diminution sélective du nombre de cellules fonctionnelles dans la zone réticulée de la corticosurrénale (Labrie et al, 1995). La particularité de cette hormone est d'être réputée pour ses effets anti-vieillissement, d'où son surnom médiatique d'« hormone de jouvence ». En effet, plusieurs études ont montré qu'une supplémentation en DHEA a de nombreux effets bénéfiques, notamment il a été montré que ce traitement améliore la masse et la force musculaire (Villareal & Holloszy, 2006), diminue l'adiposité et augmente la sensibilité à l'insuline (Jankowski et al, 2011), prévenant ainsi le risque de maladies cardiovasculaires. Cependant, on manque pour l'heure de données sur les effets indésirables associés aux prises de DHEA de longue durée. On peut s'attendre à ce qu'une supplémentation ait pour conséquence une diminution de la production endogène des hormones sexuelles (testostérone et œstrogènes), si bien que l'administration de DHEA à des sujets sains est déconseillée.

5. Le tissu adipeux

L'organe adipeux présente une grande variété cellulaire et d'importantes capacités d'adaptation fonctionnelle, grâce à sa plasticité tissulaire. Cependant, en fonction de leur localisation, les capacités des adipocytes varient de manière significative. En effet, alors que

le tissu adipeux viscéral (intrapéritonéal) a un rôle métabolique, le tissu adipeux sous-cutané (extrapéritonéal) a une fonction de réserve énergétique et le tissu adipeux périphérique (gynoïde) a, lui, un rôle structurel et de réserve énergétique pour la grossesse (Leyvraz et al, 2008).

Une augmentation progressive de l'adiposité viscérale est une des caractéristiques du vieillissement, et comme le montre plusieurs études épidémiologiques, cette accumulation de tissu adipeux viscéral (TAV) représente un facteur de risque important dans la résistance à l'insuline, le diabète, et les maladies cardiovasculaires comme l'athérosclérose (Figure 9) (Grundy, 2006 ; Britton et al, 2013; Cefalu et al, 1995). Les mécanismes potentiellement responsables de la modulation de l'action de l'insuline par le TAV incluent une augmentation de la libération d'acides gras libres (Ebbert & Jensen, 2013), et/ou une expression et une sécrétion anormale de peptides comme le facteur de nécrose tumorale TNFα (Tumor Necrosis Factor-α), l'interleukine 6 (IL-6) ou la leptine (Goossens, 2008). Ainsi, l'inflammation à bas bruit observée chez la personne âgée peut être favorisée par un excès de TAV. Cependant, certaines études réalisées chez le rat ont montré que les effets d'une accumulation de TAV ne sont pas irréversibles et qu'une ablation chirurgical de ce tissu permet de restaurer l'action de l'insuline au niveau hépatique et périphérique (Borst et al, 2005; Gabriely et al, 2002).

6. L'appareil locomoteur

L'appareil locomoteur, constitué des os, des articulations et des muscles squelettiques, est responsable du maintien de la posture et est capable d'exécuter des mouvements.

Le vieillissement va entraîner une fragilité des tissus osseux et articulaires. Tout au long de la vie, notre tissu osseux est renouvelé grâce à un équilibre dynamique entre synthèse et dégradation. Ce remodelage continu permet au tissu osseux de conserver ses propriétés biomécaniques. Lors du processus normal du vieillissement, les capacités de prolifération de l'os vont diminuer : les quantités d'os formées deviennent alors moins importantes que celles d'os résorbé (Demontiero et al, 2012). Ceci va alors entraîner une perte de tissu osseux et une fragilisation des os, aboutissant à l'ostéoporose (Sipos et al, 2009). Ce système prend encore plus d'ampleur suite aux dérèglements hormonaux résultant de la ménopause (Figure 10). D'après la Société Française de Myologie, 30 à 40 % des femmes ménopausées et plus de la moitié de celles de plus de 75 ans sont touchées par l'ostéoporose en France. Les principales conséquences sont les fractures osseuses et les tassements vertébraux réduisant l'autonomie motrice.

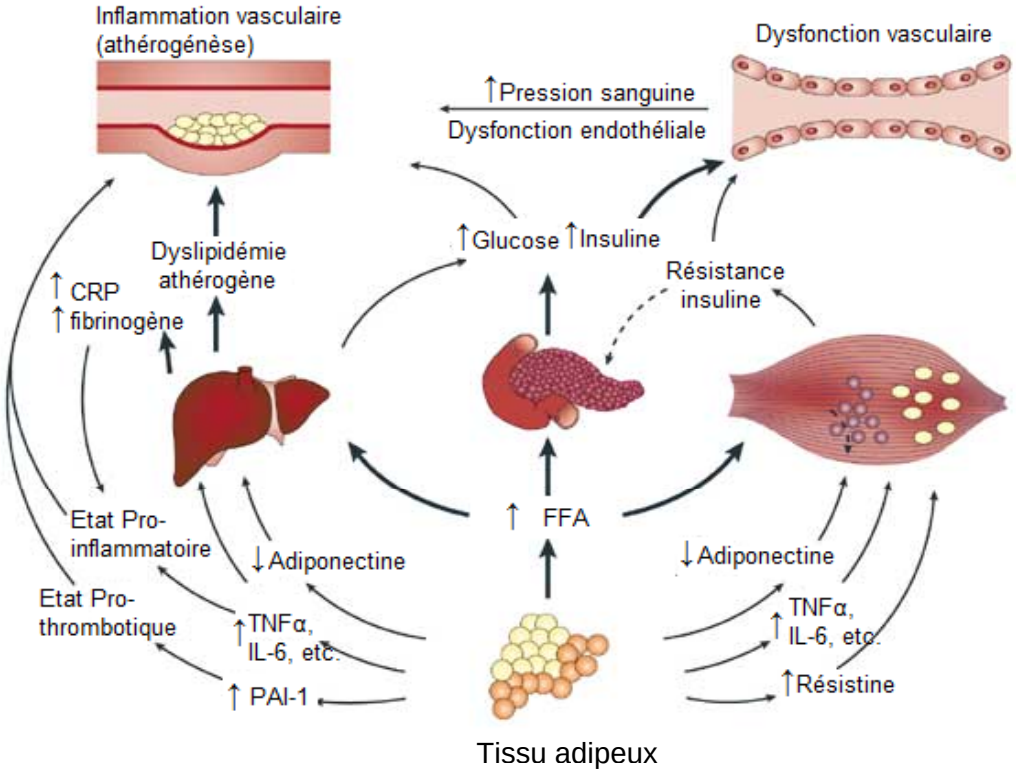

Figure 9: Effet du tissu adipeux sur l'apparition de la résistance à l'insuline et le développement de l'athérosclérose. FFA, Free fatty acid; TNFα, Tumor necrosis factor-α; IL-6, Interleukine-6; PAI-1, Plasminogen activator inhibitor-1; CRP, C-réactive protéine.
D'après: **Grundy**, 2006

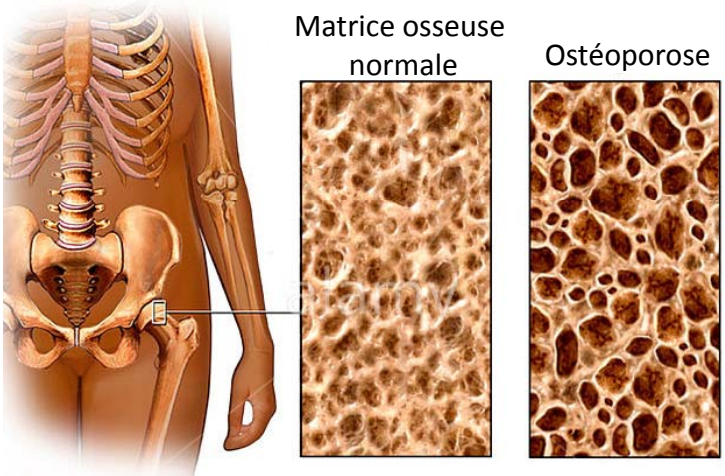

Figure 10: Illustration de l'altération de la matrice osseuse chez une femme ménopausée atteinte d'ostéoporose.
D'après: www.alamy.com

De la même façon, il apparaît avec le vieillissement une altération du cartilage articulaire : l'arthrose qui est l'une des maladies les plus courantes affectant les personnes âgées. L'arthrose se développe sous deux formes différentes, l'ostéoarthrite (OA) et l'arthrite rhumatoïde (AR). L'OA est la forme la plus courante d'arthrose et elle touche principalement les articulations des mains, des genoux et de la hanche (Bijlsma et al, 2011). Elle est caractérisée par une dégradation progressive du cartilage causée par l'augmentation de l'expression de protéoglycanes et de métalloprotéases, clivant et dégradant le collagène (Wieland et al, 2005). Contrairement à l'OA, l'AR est considérée comme étant une maladie chronique inflammatoire. Les articulations des mains, des poignets, des genoux et des pieds sont principalement affectées avec des symptômes incluant enflures, douleurs, mouvements limités et déformations des membres touchés (Scott et al, 2010). L'arthrite rhumatoïde implique une réponse du système immunitaire inné et adaptatif, où une production excessive de facteurs pro-inflammatoires (TNFα ou IL-1) par les macrophages active la sécrétion de cytokines, chémokines, facteurs de croissance et prostaglandines par les chondrocytes, les fibroblastes ou par les leucocytes (McInnes & Schett, 2007).

En plus de la fragilité des tissus osseux et articulaires, le vieillissement du muscle squelettique est sans doute une des conséquences majeures de la perte d'autonomie et de l'augmentation de la morbidité chez la personne âgée. La structure, le fonctionnement ainsi que les altérations du muscle squelettique lors du vieillissement seront développés dans les chapitres suivants.

Chapitre II : Le muscle squelettique, machine contractile et acteur métabolique

Le muscle squelettique est un organe hautement spécialisé doué de capacités régénératives et de propriétés d'excitabilité, de contraction, d'élasticité et de plasticité. Ces propriétés lui permettent d'assurer des fonctions importantes comme la mobilité et le maintien de la posture. Le muscle squelettique appartient, avec le muscle cardiaque, à la famille des muscles striés et est sous contrôle du système nerveux central. Cet organe est composé de fibres musculaires regroupées sous forme de faisceaux et entourées par la matrice extracellulaire. Il existe différents types de fibres caractérisées par leur métabolisme oxydatif et/ou glycolytique, leur permettant de produire un mouvement plus ou moins puissant et rapide, adapté à une situation particulière. Le tissu vasculaire, assurant un apport d'oxygène et de nutriments, ainsi que les cellules satellites, responsables de la régénération musculaire, sont des constituants majeurs du muscle squelettique et sont nécessaires au bon fonctionnement de ce tissu.

Cependant, le muscle squelettique n'a pas qu'une fonction de contraction, c'est également un acteur métabolique important. En effet, le muscle est une réserve de protéines facilement hydrolysable en acides aminés afin de lutter contre les agressions de l'organisme. De plus, le muscle est un site d'homéostasie du glucose et est également fortement impliqué dans le métabolisme lipidique.

Ce chapitre s'attache donc à décrire la structure et le fonctionnement du muscle squelettique mais également son rôle essentiel dans le métabolisme énergétique.

I. Structure et fonctionnement du muscle strié squelettique

1. Structure de la fibre musculaire

a- La myofibre : entité cellulaire du muscle

Le muscle squelettique est constitué de faisceaux de cellules plurinucléées : les fibres musculaires, ou myofibres. Chacune d'entre elles renferme plusieurs centaines de noyaux

aplatis disposés à la périphérie du cytoplasme et accolés à la membrane sarcoplasmique, ou sarcolemme. Ces fibres ont un diamètre compris entre 5 à 100 µm et une longueur très variable de 1 mm à plusieurs centimètres. Elles sont regroupées en faisceaux imbriqués les uns dans les autres et maintenus ensemble par différentes couches de tissu conjonctif : l'épimysium, le périmysium et l'endomysium (Turrina et al, 2013) (Figure 11).

Le volume intracellulaire de la myofibre est presque totalement occupé par les myofibrilles. Ce sont des structures tubulaires allongées d'un diamètre de 1 à 2 µm, et constituées de myofilaments disposés selon une organisation géométrique extrêmement rigoureuse.

b- Sarcolemme et réticulum sarcoplasmique : un réseau membranaire

La membrane plasmique qui délimite la fibre musculaire est dénommée le sarcolemme. C'est une structure continue qui parcourt la fibre musculaire et contribue à l'intégrité mécanique. La force de contraction, qui est transmise par les myofibrilles, traverse le sarcolemme en direction de la matrice extracellulaire, le sarcolemme est donc exposé à un stress mécanique intense nécessitant une structure forte. Il présente une particularité unique dans le tissu musculaire puisqu'il se prolonge vers l'intérieur de la cellule pour former un réseau de tubules transversaux, aussi nommés tubules T (Ishikawa, 1968).

Le sarcolemme entoure le réticulum sarcoplasmique (RS), dont la fonction principale est de stoker et de relarguer le calcium (Ca^{2+}), ce qui correspond respectivement à la relaxation et la contraction du muscle. Le réticulum sarcoplasmique est formé par des canaux longitudinaux qui débouchent dans des cavités, appelées citernes terminales. Ces citernes entourent les tubules T et l'ensemble forme la « Triade de Pallade » (Al-Qusairi & Laporte, 2011) (Figure 12). Cette structure est impliquée dans la transmission du signal nerveux permettant la contraction des myofibrilles *via* la libération du calcium contenu dans les cavités du RS. Ainsi, cette triade joue un rôle important dans le contrôle de la contraction musculaire (Rossi et al, 2008).

Le sarcolemme possède également une région spécialisée qui est la jonction neuromusculaire ou plaque motrice. C'est une synapse particulière permettant le contact étroit entre la fibre musculaire et la terminaison nerveuse (Takamori, 2012). Il est à noter

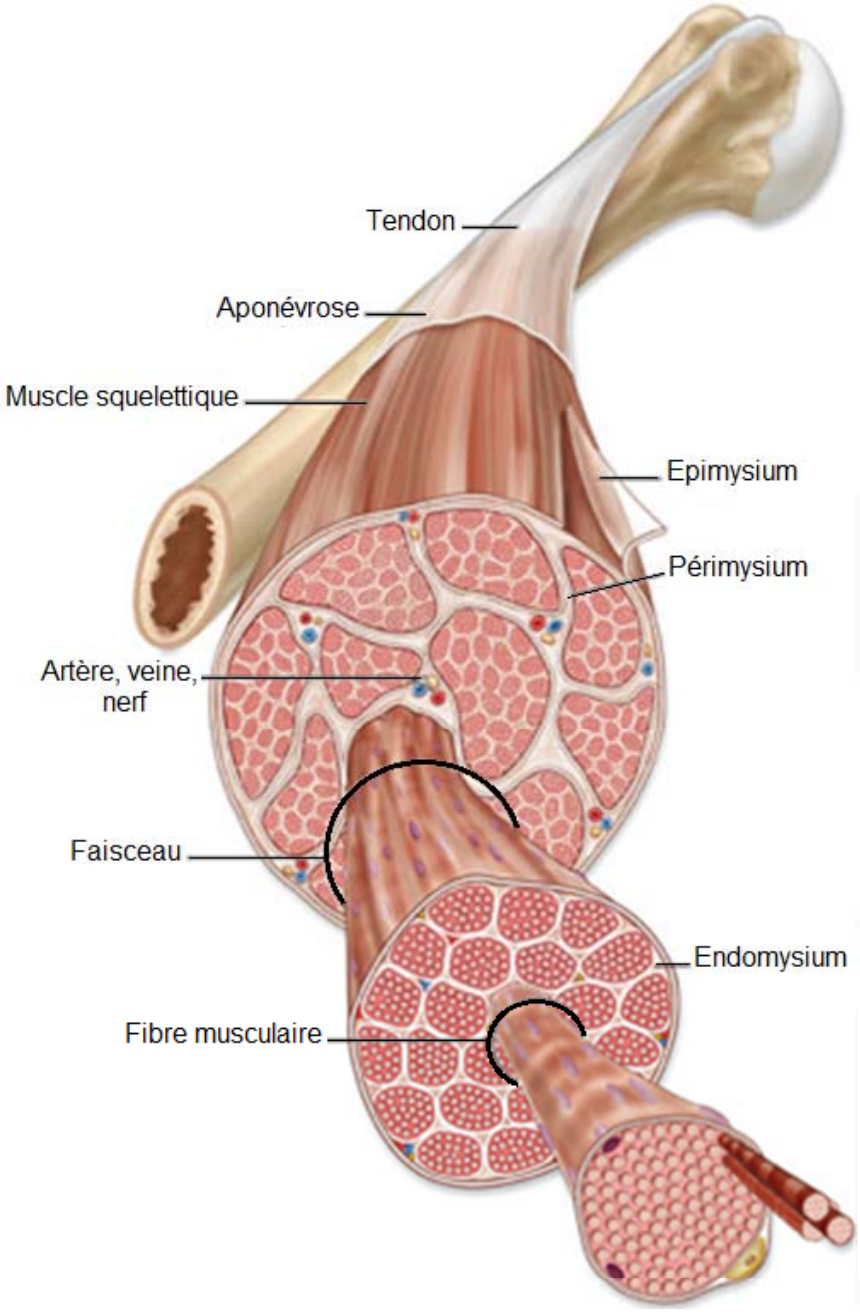

Figure 11: Structure du muscle squelettique
D'après: **Williams & Warwick**. Gray's Anatomy. Thirty-seventh edition. Churchill Livingstone. ISBN 0-443-04177-6.

que chaque fibre est sous le contrôle d'un seul motoneurone. Cependant, un même motoneurone peut innerver plusieurs fibres musculaires.

c- La matrice extracellulaire

La matrice extracellulaire (MEC) est un assemblage complexe de protéines interactives en équilibre dynamique qui commandent l'organisation des tissus et modulent leurs fonctions. Elle lie un certain nombre de facteurs de croissance et régule l'adhérence des cellules, la migration cellulaire et diverses voies de transduction régissant de nombreuses fonctions cellulaires. Différents auteurs ont décrit la structure, la morphologie ainsi que la composition de cette matrice (Mayne & Sanderson, 1985 ; McCormick et al, 1994; Purslow & Duance, 1990). Chaque muscle est délimité par l'épimysium, le périmysium sépare les faisceaux de fibres musculaires et chaque fibre est entourée par l'endomysium (Borg & Caulfield, 1980 ; Turrina et al, 2013) (Figure 11). Enfin, chaque cellule est entourée par de la matrice extracellulaire, la lame basale. Initialement, la membrane basale était décrite comme une structure statique, ayant uniquement un rôle de support mécanique. Toutefois, les composés de cette membrane possèdent en fait des rôles actifs dans les processus du développement et de régénération (Sanes, 2003).

Les protéines de la matrice extracellulaire représentent entre 10 et 15 % des protéines musculaires, la protéine principale étant le collagène qui peut représenter 70 à 80 % des protéines du tissu conjonctif. Il a été dénombré sept types de collagènes au sein des muscles : types I, III, IV, V, VI, XII et XIV (Listrat et al, 1999). L'élastine est une autre protéine également présente dans la matrice extracellulaire. Cette protéine est moins largement distribuée que le collagène et forme le cœur des fibres élastiques. Les protéoglycanes (PGs) jouent un rôle important dans les liaisons entre les éléments fibreux de la matrice extracellulaire (Scott, 1990). Cette dernière contient également des protéines non-collagéniques comme la fibronectine, la laminine, la vitronectine ou encore les tenascines, ainsi que diverses glycoprotéines de surface comme les intégrines. Ces protéines jouent un rôle essentiel dans l'adhérence des cellules à leur substrat, en conditionnant leurs déplacements et leurs fonctions de synthèse.

Le rôle fonctionnel des composants du tissu conjonctif intramusculaire reste encore à préciser mais il semblerait qu'ils auraient un rôle central lors de la croissance et dans la

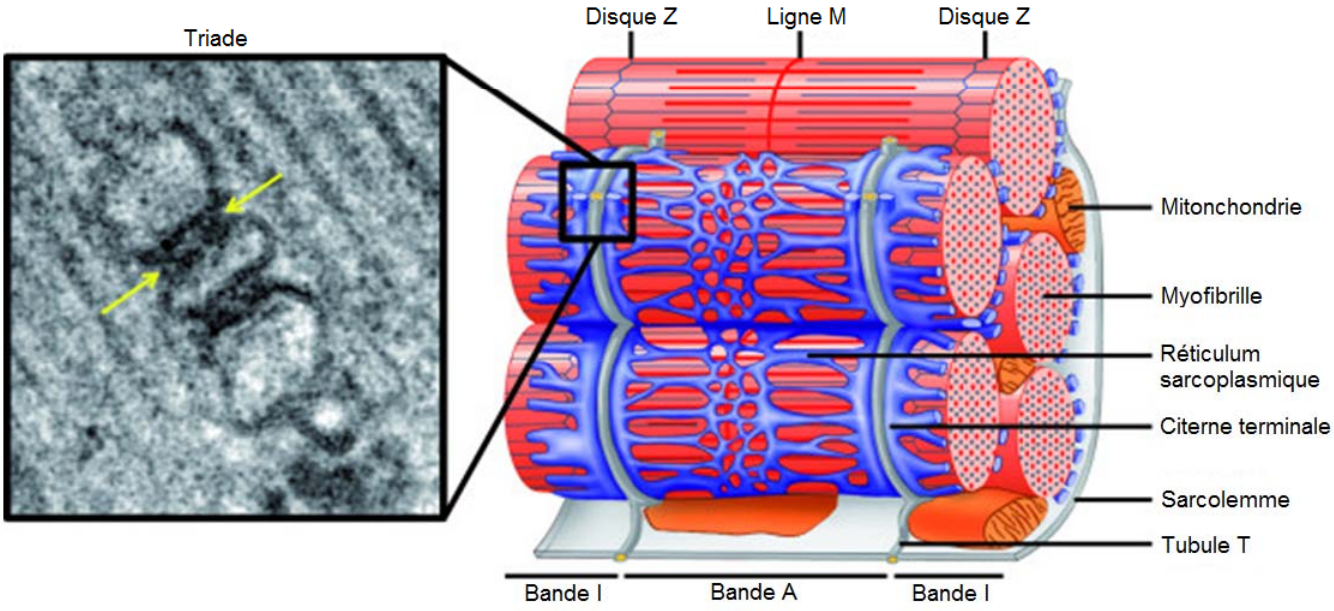

Figure 12: Organisation de la « Triade de Pallade » dans le muscle squelettique
D'après: **Al-Qusairi & Laporte**, 2011.

transmission des signaux mécaniques, nerveux ou hormonaux aux cellules musculaires. En effet, le tissu conjonctif intramusculaire est un support mécanique pour les nerfs, les neurones, ainsi que pour les vaisseaux sanguins de chaque fibre. La matrice extracellulaire du muscle squelettique est également essentielle pour la transmission de la force et l'élasticité du muscle squelettique. La structure, les propriétés biochimiques et la fonction de cette matrice démontrent une balance dynamique entre sa formation, son remodelage et sa dégradation, en particulier en réponse à une charge mécanique (Kjaer, 2004). De plus, ce tissu détermine l'étendue des déformations auxquelles pourra être soumis la surface des myofibres lors d'une contraction (Purslow, 2002).

d- Autres composants du muscle

- Les vaisseaux sanguins :

Le sang est transporté dans l'organisme par un réseau de vaisseaux sanguins (Pugsley & Tabrizchi, 2000):
- les artères qui transportent le sang en provenance du cœur et qui se ramifient en vaisseaux de plus en plus petits pour donner les artérioles
- les veinules qui convergent et fusionnent pour former des veines de plus en plus grosses amenant le sang vers le cœur
- les capillaires sanguins qui permettent les échanges entre le sang et le liquide interstitiel dans lequel baignent les cellules.

Le muscle squelettique possède un réseau de capillaires particulièrement dense présent dans la matrice extracellulaire. Contrairement aux artères et aux veines, les parois des capillaires ne sont formées que de cellules endothéliales. Ils se regroupent en réseaux pour former les lits capillaires composés d'une dérivation vasculaire et de capillaires vrais (Merlen, 1976 ; Tortora & Grabowski, 2001) (Figure 13). Ces structures particulières relient une artériole à une veinule et permettent des échanges de substances entre le sang et les cellules musculaires : O_2, CO_2, nutriments, métabolites, hormones, etc. En fonction de l'état du muscle (en repos ou lors d'un exercice physique), les besoins en nutriments varient considérablement. Ainsi, le volume de sang circulant dans un lit capillaire est fortement

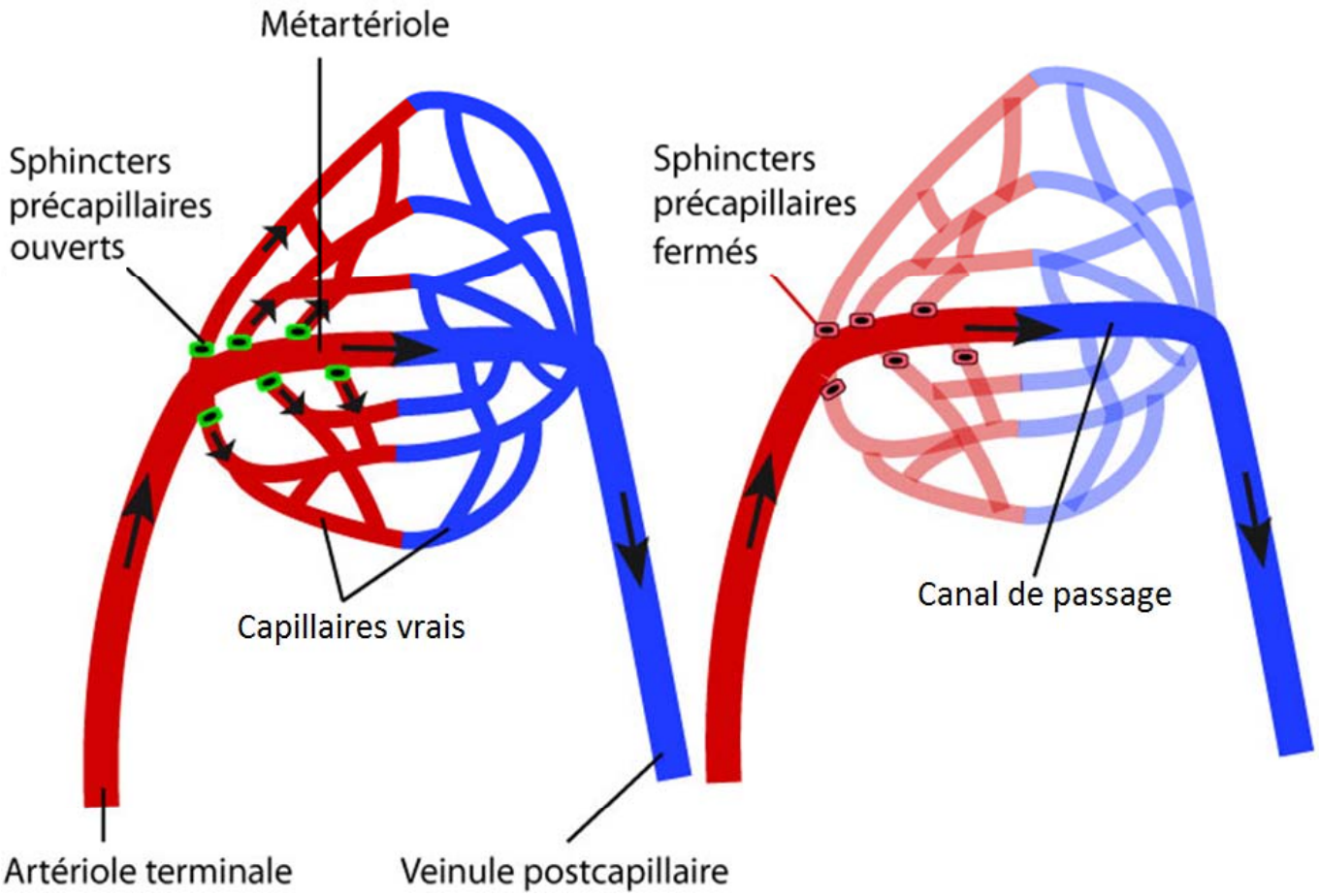

Figure 13: Représentation schématique d'un lit de capillaire composée d'une dérivation capillaire, formée par une métartériole et un canal de passage, et de capillaires vrais. Des sphincters précapillaires entourent la racine de chaque capillaire vrai afin de contrôler l'écoulement de sang.
A gauche, les sphincters sont ouverts et le sang emprunte l'ensemble des capillaires sanguins et participe aux échanges avec le tissu musculaire. A droite, les sphincters sont fermés et la circulation du sang se limite à la métartériole et au canal de passage, le sang contourne alors les capillaires vrais et les cellules.
D'après: **Tortura & Grabowski**, 2000.

contrôlé *via* deux systèmes indépendants : les neurofibres vasomotrices et les conditions chimiques locales (Moppett, 2012).

- Les nerfs et unités motrices:

L'activité normale d'un muscle squelettique dépend de son innervation. Les fibres musculaires sont innervées par des fibres motrices, aussi appelées motoneurones. La structure de base autour de laquelle s'articule la physiologie musculaire est l'unité motrice (Duchateau & Enoka, 2011). Celle-ci est formée par un motoneurone situé dans la moelle épinière, son prolongement (axone) qui chemine dans le nerf périphérique et l'ensemble des fibres musculaires que le motoneurone innerve (Figure 14) (Mekrami & Brignol, 2003). Chaque motoneurone innerve plusieurs fibres musculaires qu'il active de façon synchrone. Cependant, chaque axone se divise en un certain nombre de ramifications, chacune d'elles innervant une seule fibre musculaire.

Dans un muscle, on identifie différents types d'unités motrices selon les propriétés de leurs motoneurones et de leurs fibres musculaires. Il existe les unités motrices S (Slow) induisant une contraction lente, et les unités motrices F (Fast) aboutissant à une contraction rapide (Clamann, 1993). Les unités motrices F sont, à nouveau, subdivisées en trois groupes suivant leur capacité de résistance à la fatigue :

- FR (*Fatigue Resistance*): résistance à la fatigue.
- FF (*Fast Fatigable*) : fatigabilité
- FI (*Fatigue Intermédiaire*) : résistance à la fatigue intermédiaire entre les unités motrices FR et FF.

Dans notre organisme, nos muscles striés squelettiques sont dits hétérogènes, c'est-à-dire qu'ils sont composés d'un mélange de ces quatre types d'unités motrices (S, FR, FF et FI), mais en proportions variables selon les muscles. Les muscles de type posturaux sont plus riches en unités motrices de type lentes (ex : le muscle *soleus*), alors que les muscles impliqués dans un mouvement dynamique ont une forte proportion d'unités motrices de type rapides (ex : le muscle *vastus lateralis*). Ainsi, en fonction du type de contraction nécessaire, un recrutement préférentiel de certaines unités motrices sera effectué (Hodson-Tole & Wakeling, 2009).

- Les cellules satellites :

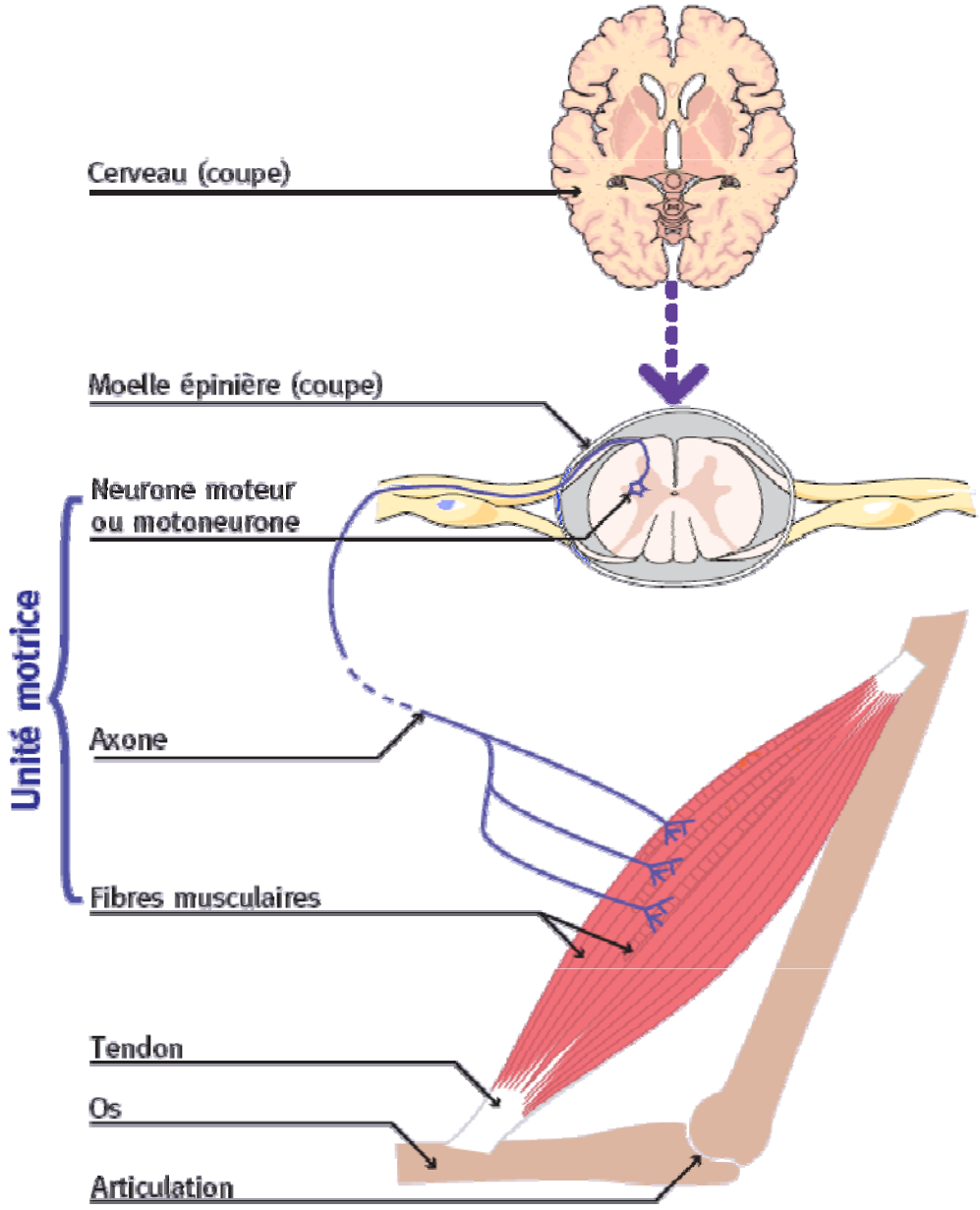

Figure 14: Contrôle de l'activité du muscle squelettique par le système nerveux central.
D'après: **Mekrami & Brignol**, 2003.

La définition des cellules satellites a été donnée pour la première fois par Mauro en 1961 (Mauro, 1961). Elle repose sur la localisation de ces cellules, juxtaposées aux fibres musculaires sous la lame basale où elles sont, dans le muscle adulte, dans un état quiescent. Le rôle des cellules satellites est d'assurer la croissance des fibres musculaires en maintenant le rapport entre le nombre de noyaux et le volume des fibres. De plus, elles sont également responsables de la régénération des fibres musculaires après une lésion. Cette régénération du muscle squelettique passe par plusieurs étapes où la cellule satellite va exprimer différents marqueurs spécifiques de sont état :

- avant la lésion : la cellule satellite est en état de quiescence où elle arrête de se diviser en G0 et sort du cycle cellulaire. A ce stade, la cellule va exprimer des marqueurs cellulaires spécifiques comme le facteur de transcription Pax7 (Seale et al, 2000).

- après la lésion : suite aux signaux issus des dommages infligés au muscle squelettique, les cellules satellites quiescentes sont activées, elles entrent dans le cycle cellulaire et prolifèrent, permettant une expansion de la population de cellules myogéniques. Parmi les facteurs provoquant la sortie de la quiescence, on retrouve différents facteurs de croissance comme IGF-I (Insulin like Growth Factor I), FGF (Fibroblast Growth Factor), TGF-β (Transforming Growth Factor β) ou HGF (Hepatocyte Growth Factor), ou encore des interleukines comme l'IL-6 (Allen & Boxhorn, 1989 ; Charge & Rudnicki, 2004). Les cellules satellites activées sont caractérisées par une forte expression des facteurs MRFs (Muscle Regulatory Factors) comme MyoD ou Myf5 (Cooper et al, 1999). Cette phase prolifération est ensuite suivie d'une phase de différenciation terminale, puis d'une fusion des myoblastes au niveau des fibres musculaires endommagées afin de les réparer ou d'en former de nouvelles (Figure 15) (Charge & Rudnicki, 2004). Cette différentiation terminale des myoblastes est, elle, caractérisée par la surexpression d'autres facteurs MRFs comme la myogénine ou MRF4 (McKay et al, 2008). De plus, pour maintenir le potentiel de régénération, la réserve de cellules satellites doit se renouveler continuellement. Ainsi, à chaque activation, une partie des cellules va se différencier pour réparer les fibres endommagées tandis qu'une autre partie va participer au renouvellement de la réserve.

2. Organisation moléculaire de la myofibrille

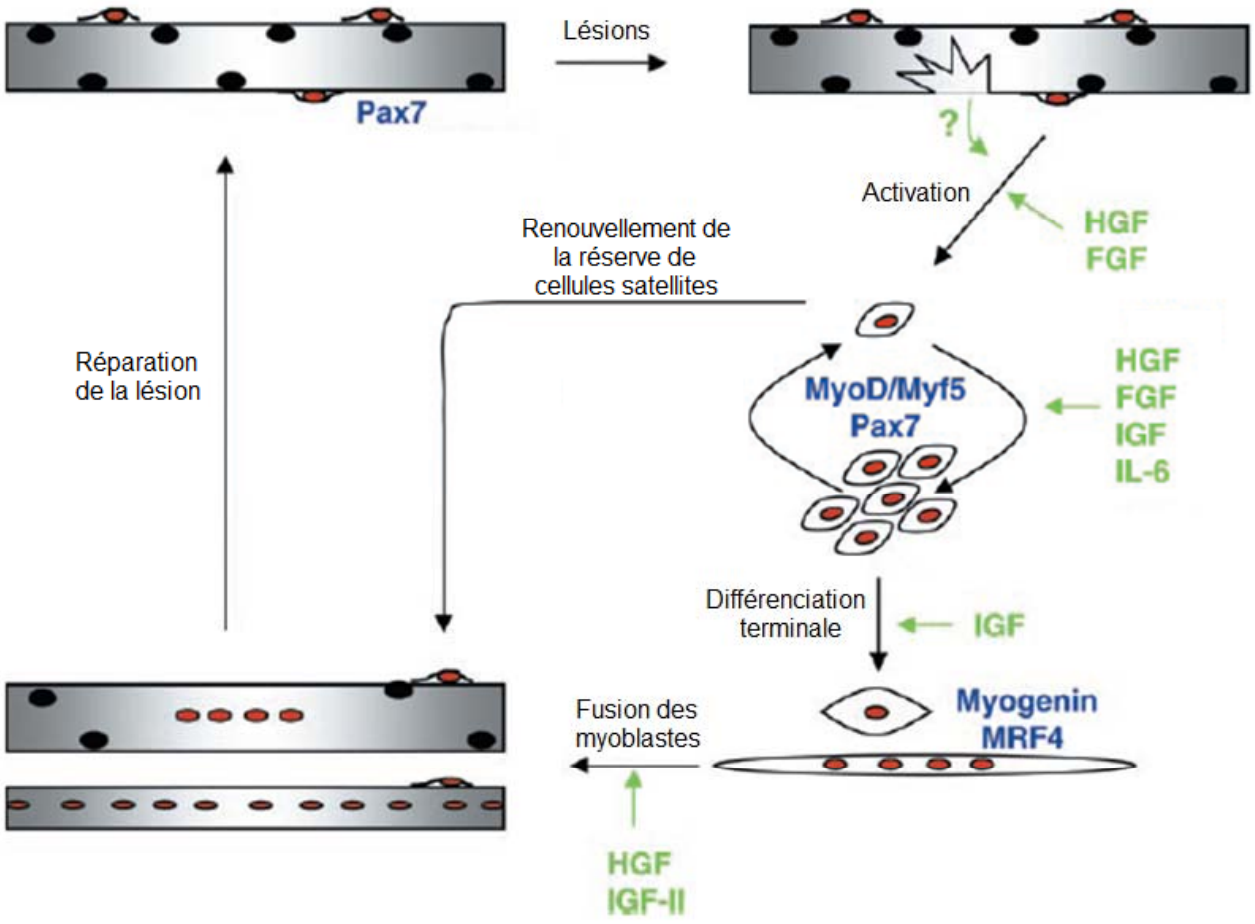

Figure 15: Représentation schématique des évènements moléculaires régulant l'activation des cellules satellites durant la régénération d'une fibre musculaire suite à une lésion.
D'après: **Chargé & Rudnicki**, 2004.

a- Le sarcomère : unité contractile

En microscopie photonique, les myofibrilles ont un aspect strié dû à des indices de réfraction différents des diverses zones des fibres (Figure 16 A). Ainsi, on peut observer des bandes sombres dites bandes A (Anisotrophe) étant coupées en leur milieu par une rayure plus claire : la zone H (de l'allemand Heller signifiant « plus pâle »), seulement visible lorsque les fibres musculaires sont au repos. Cette zone H est interrompue en son milieu par une zone plus sombre : la ligne M (de l'allemand Mittel, « centre »). On observe également des bandes claires dites bandes I (Isotrophe) au centre desquelles se trouve une région plus foncée : le disque Z (de l'allemand Zwischen, « entre »). La région d'une myofibrille comprise entre deux disques Z, d'une longueur d'environ 2,4 µm, constitue le sarcomère, unité contractile de la fibre musculaire (Bloom & Fawcett, 1962 ; Sciote & Morris, 2000) (Figure 16 B).

Ce sarcomère est placé en série et contient deux types de myofilaments : l'un épais, de myosine, et l'autre fin, d'actine. Au niveau de cette structure, les filaments fins sont attachés de part et d'autre d'un matériel protéique essentiel, le disque Z, tandis que les filaments épais s'attachent au niveau de la ligne M. C'est le chevauchement des filaments fins et épais qui constituent la bande A. Au cours de la contraction musculaire, le glissement des filaments les uns sur les autres entraîne une diminution de la zone H, correspondant à un raccourcissement des sarcomères. En plus du réseau d'acto-myosine, le sarcomère contient également deux autres réseaux de filaments : la titine et la nébuline.

b- Les filaments fins et épais

- Les filaments fins

Ils ont un diamètre d'environ 7 nm et sont constitués de plusieurs types de molécules dont les principales sont l'actine, la tropomyosine et la troponine. L'alpha actine monomérique (ou actine globulaire) se polymérise pour former des filaments (actine filamenteuse) composés de deux chaînes linéaires qui s'enroulent l'une autour de l'autre pour former une double hélice (Figure 16 C). La tropomyosine va se lier au niveau des sillons des filaments d'actine et ainsi bloquer les liaisons avec la myosine lorsque que la fibre est au repos. La contraction est alors inhibée. De plus, un complexe de troponines (I, T

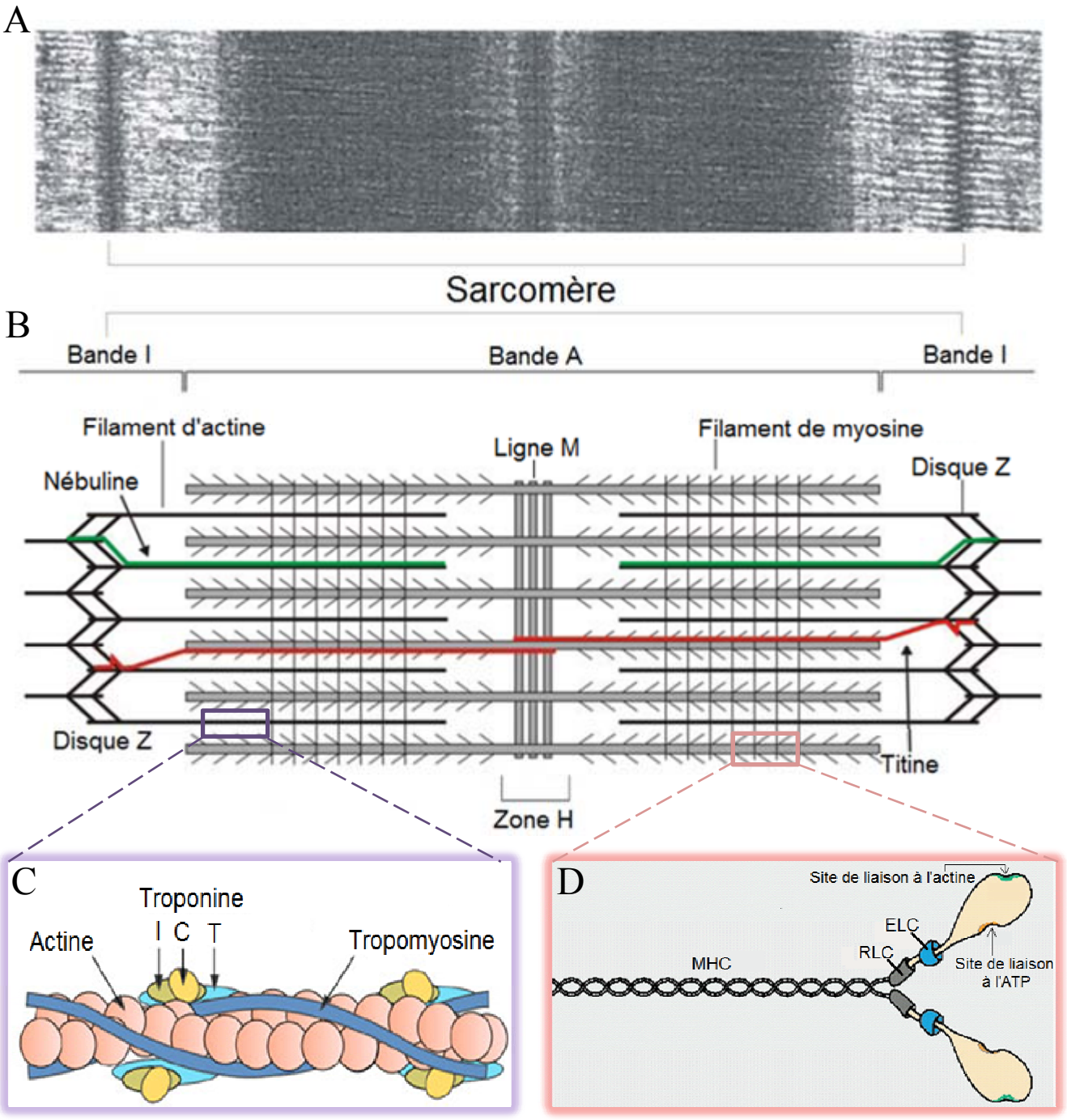

Figure 16: Structure du sarcomère, des filaments d'actine et des filaments épais de myosine dans un muscle squelettique.
A. Photographie en microscopie électronique d'une coupe longitudinale de muscle squelettique
B. Schéma montrant les composants principaux du sarcomère
C. Schéma d'un filament fin d'actine
D. Schéma d'un filament épais de myosine
D'après: **Al-Qusairi & Laporte**, 2011; **Gordon** et al, 2000

et C) est attaché au système actine-tropomyosine afin de contrôler et réguler les interactions actine-myosine (Gomes et al, 2002). En effet, la troponine I et T inhibent l'activité ATPasique de la myosine, en présence de tropomyosine. La troponine C, elle, fixe le calcium et par conséquent joue un rôle déterminant dans la régulation calcique de la contraction.

- Les filaments épais

Ils sont constitués de plusieurs molécules de myosine formées de deux chaînes lourdes (MHC, Myosin Heavy Chain). Chaque MHC possède une queue, ainsi qu'une tête globulaire où s'associent deux chaînes légères (MLC, Myosin Light Chain) dont l'une est dite « essentielle » (ELC, Essential Light Chain) et l'autre « régulatrice » (RLC, Regulatory Light Chain) (Figure 16 D) (Rayment et al, 1993). On trouve de nombreuses isoformes de myosines lourdes et légères, dont l'expression varie suivant le type de muscle et est adaptée aux forces développées. Des protéines liées aux myosines (MyBP, Myosin Binding Proteins) s'associent aux filaments épais et participent au bon déroulement de la polymérisation de la myosine (Okagaki et al, 1993).

c- Les protéines du disque Z

Le disque Z apparait comme une ligne fine et dense formant les bords du sarcomère dans les muscles striés. Il révèle un réseau de filaments réticulés qui transmettent la tension et abritent d'innombrables protéines ayant diverses fonctions (Luther, 2009). Au niveau du disque Z, les extrémités dites « barbues » des filaments d'α-actine s'entrecroisent et s'ancrent *via* l'α-actinine, une protéine ubiquitaire que l'on trouve dans les cellules eucaryotes (Blanchard et al, 1989). En plus de son rôle structural, il est connu que l'α-actinine a un rôle majeur dans la signalisation cellulaire et dans l'ancrage d'autres protéines du disque Z (Sjoblom et al, 2008).

En effet, en plus des filaments d'actine, beaucoup d'autres protéines vont s'ancrer au niveau de ce disque Z parmi lesquelles on trouve la titine, la nébuline ou la protéine CapZ (Figure 17) :

- Titine : cette protéine, aussi appelée connectine, correspond au troisième réseau de filament (Granzier & Labeit, 2005). Les molécules de titine sont des protéines élastiques

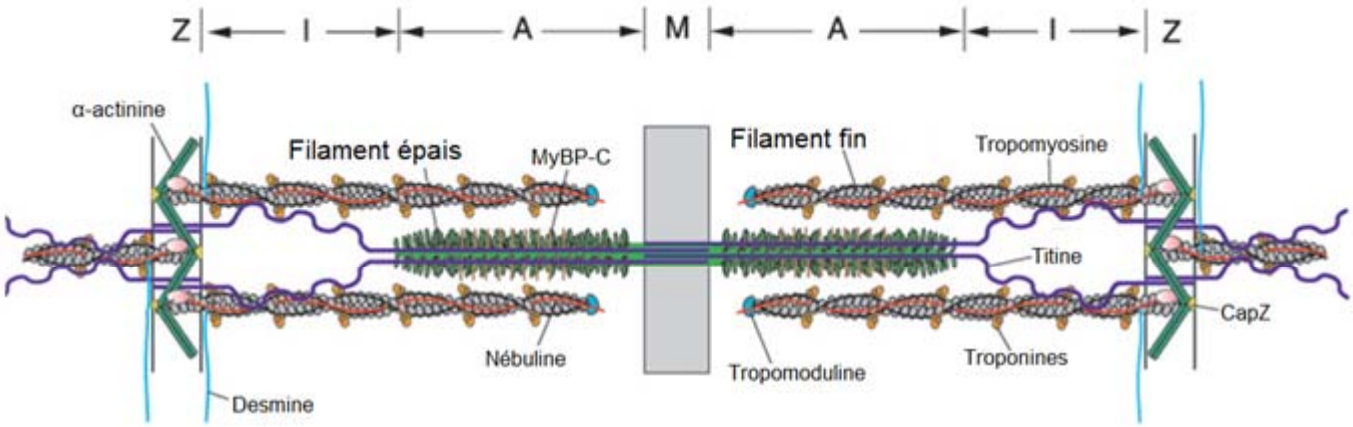

Figure 17: Représentation schématique de l'ancrage des filaments fins et épais aux disques Z *via* l'intermédiaire de plusieurs protéines parmi lesquelles on retrouve l'α-actinine, la titine, la nébuline et CapZ.
Ce schéma montre également la présence de la desmine au niveau du disque Z. Cette protéine appartient aux costamères et permet l'ancrage des filaments d'acto-myosine au niveau du sarcolemme de la cellule.
D'après: **McElhinny** et al, 2003

géantes d'environ 3000 kDa capables d'interagir avec de nombreuses protéines comme l'actine, la myosine ou la calmoduline. Ceci lui confère de nombreuses fonctions, que ce soit au niveau de la contraction, de la gestion des forces mécaniques, ou lors de la myofibrillogenèse. De par ces nombreuses interactions, la titine contrôle l'assemblage des protéines sarcomériques régulant l'élasticité du sarcomère. Elle permet, notamment, aux filaments fins et épais d'être liés au disque Z et à la ligne M.

- Nébuline : c'est une protéine géante du sarcomère qui se fixe sur le disque Z *via* son extrémité N-terminale. Elle s'étend sur toute la longueur des filaments d'α-actine où elle forme un filament composite avec le complexe actine/tropomyosine/troponine. La nébuline joue un rôle important dans l'assemblage, la structure et la fonction du disque Z, et sa longueur semble dicter celle des filaments d'actine (Kruger et al, 1991 ; Pappas et al, 2008). De plus, cette protéine participerait à la régulation de la contraction musculaire en inhibant l'activité ATPasique de la myosine (McElhinny et al, 2003).

- CapZ : cette protéine est localisée au niveau du disque Z où elle se lie à l'α-actinine, formant un complexe d'ancrage des filaments fins d'α-actine (Papa et al, 1999). En liant les extrémités « barbues » des filaments d'actine, CapZ permettrait d'organiser et d'aligner ces filaments et ainsi aurait un rôle dans la régulation de la dynamique de l'actine. En plus de son interaction avec l'α-actinine, Pappas et *al.* en 2008 ont montré que la localisation de CapZ au niveau du disque Z est permise par sa liaison avec la nébuline.

d- Costamères et jonctions myotendineuses

Pour transmettre efficacement la force, le cytosquelette contractile doit être attaché au sarcolemme des myofibrilles. Les muscles striés squelettiques contiennent deux systèmes de liaison membranaire possédant des similarités structurales et moléculaires : les costamères et les jonctions myotendineuses (Figure 18).

➢ Les costamères

Les costamères sont des structures membranaires permettant l'attachement de l'appareil contractile à la membrane basale des fibres musculaires et qui s'ancrent au niveau du disque Z et de la ligne M (Pardo et al, 1983 ; Porter et al, 1992). Ces structures

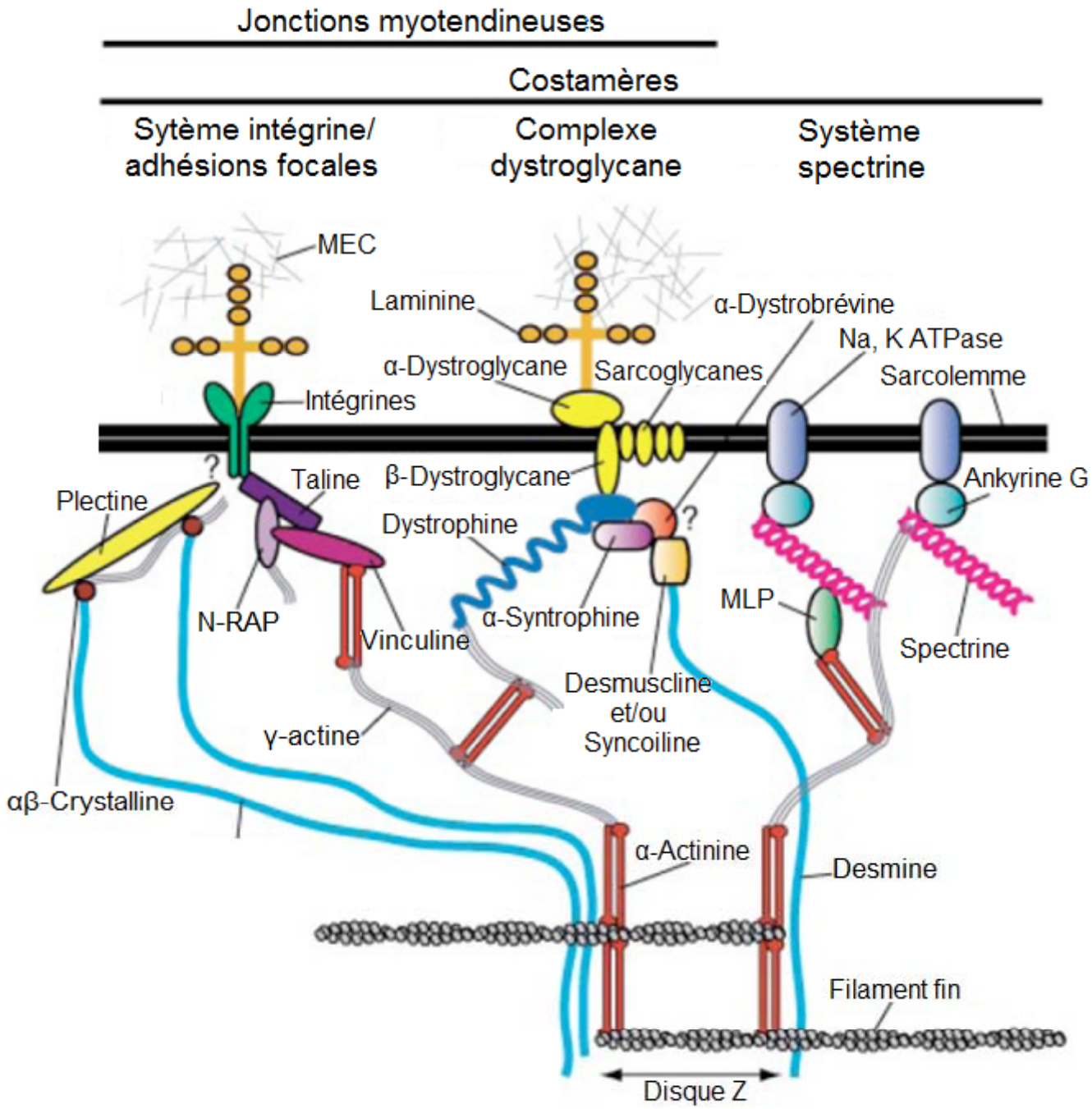

Figure 18: Structure et composants des costamères et des jonctions myotendineuses au niveau du sarcolemme.
D'après: **Clark** et al, 2002.

coordonnent la transduction de la force contractile du disque Z vers le sarcolemme, où la force est transmise latéralement aux extrémités du muscle. Les costamères permettent également de conserver l'intégrité structurale de la membrane au cytosquelette durant la contraction. Ils sont composés de trois réseaux distincts mais liés entre eux : le système intégrines/adhésions focales, le complexe dystroglycane, et le système spectrine.

Les intégrines sont des protéines transmembranaires hétérodimériques qui permettent l'attachement du cytosquelette d'actine à la matrice extracellulaire. Chaque sous-unité possède un domaine extracellulaire large qui se lie directement aux composants de la matrice, ainsi qu'un domaine cytoplasmique qui interagit avec le cytosquelette d'actine *via* son association avec plusieurs protéines comme la taline, la vinculine ou l'α-actinine (Calderwood et al, 2000). En plus de leur rôle dans l'attachement du cytosquelette, les intégrines jouent également un rôle dans la signalisation. Cette double fonction leur permet de détecter un stress mécanique et d'activer les voies de transduction du signal intracellulaire, produisant des changements dans la transcription des gènes et une réorganisation du cytosquelette (Shyy & Chien, 1997).

Le complexe dystroglycane (DGC) est un complexe de protéines multimériques dont le noyau est composé de dystrophine. Cette protéine permet la connexion entre la matrice extracellulaire environnante, le sarcolemme et le cytosquelette sous-jacent en liant la β-dystroglycan à l'actine costamérique. En absence de dystrophine, l'actine costamérique n'est plus étroitement associée avec la membrane basale des myofibres, ce qui résulte en un affaiblissement du sarcolemme, le rendant susceptible à des dommages induits lors de la contraction (Petrof et al, 1993). D'autres composants du DGC ont un rôle essentiel dans la stabilisation du cytosquelette musculaire. C'est le cas des sarcoglycanes, protéines membranaires s'associant aux β-dystroglycanes qui sont, eux, des récepteurs transmembranaires capables de lier la laminine. Enfin, le DGC inclue également des molécules adaptatrices que sont l'α-dystrobrévine et les syntrophines. Ces protéines servent de connexion entre la dystrophine et la signalisation de l'oxyde nitrique. Le complexe dystroglycane a donc un rôle structural mais il participe également à la signalisation cellulaire.

Le système spectrine est abondant au niveau des costamères, cependant sa fonction dans cette jonction est encore peu connue (Porter et al, 1992). Les dimères de spectrines sont associés à l'ankyrine G, aux ATPases sodium et potassium dépendantes et à la MLP

(Muscle LIM Protein), ce qui assure l'ancrage à la membrane. On retrouve des hétérodimères α et β de spectrine avec la MLP au niveau des disques Z, alors que les hétérodimères β sont localisés dans la ligne M. Le système spectrine participe donc à un réseau allant de la membrane au sarcomère.

- Les jonctions myotendineuses

Les jonctions myotendineuses sont des structures uniques du muscle squelettique qui constituent le point terminal des myofibrilles. C'est au niveau de ces jonctions que les forces générées par la contraction des myofibrilles sont transmises au travers du sarcolemme pour agir sur le tendon (Charvet et al, 2012). Tout comme les costamères, elles possèdent un système d'intégrines ainsi que le complexe dystroglycane, seul le système spectrine étant absent (Bao et al, 1993). Taline, vinculine et α7 intégrines sont également très enrichies dans ces structures.

3. La contraction musculaire

Dans les sarcomères au repos, les filaments fins et épais se chevauchent partiellement au niveau de la bande A. Au cours de la contraction, la longueur des sarcomères diminue, les disques Z successifs se rapprochent, et la zone H et la bande I rétrécissent. Or que ce soit durant la contraction ou à l'état de repos, la longueur de la bande A ainsi que celle des filaments fins et épais reste inchangée. Suite à cette observation, Huxley en 1957 propose la théorie « des filaments glissants », où le glissement des filaments fins le long des filaments épais est produit par l'alternance d'attachement et de détachement de la tête de myosine au filament d'actine (Huxley, 1957) (Figure 19). On peut distinguer plusieurs phases au cours de la contraction d'une cellule musculaire:

- Le couplage excitation/contraction : l'arrivée d'un potentiel d'action dans la terminaison nerveuse du motoneurone entraîne la libération d'acétylcholine dans la fente synaptique. Ce neuromédiateur va se fixer au niveau de son récepteur, dont l'ouverture déclenche la dépolarisation locale de la membrane post-synaptique musculaire. Ceci génère alors un potentiel d'action qui se propage le long du sarcolemme jusqu'à l'intérieur de la fibre *via* les tubules T. Cette dépolarisation entraîne alors l'ouverture de deux types de récepteurs canaux couplés et présents au niveau de la triade : le récepteur aux

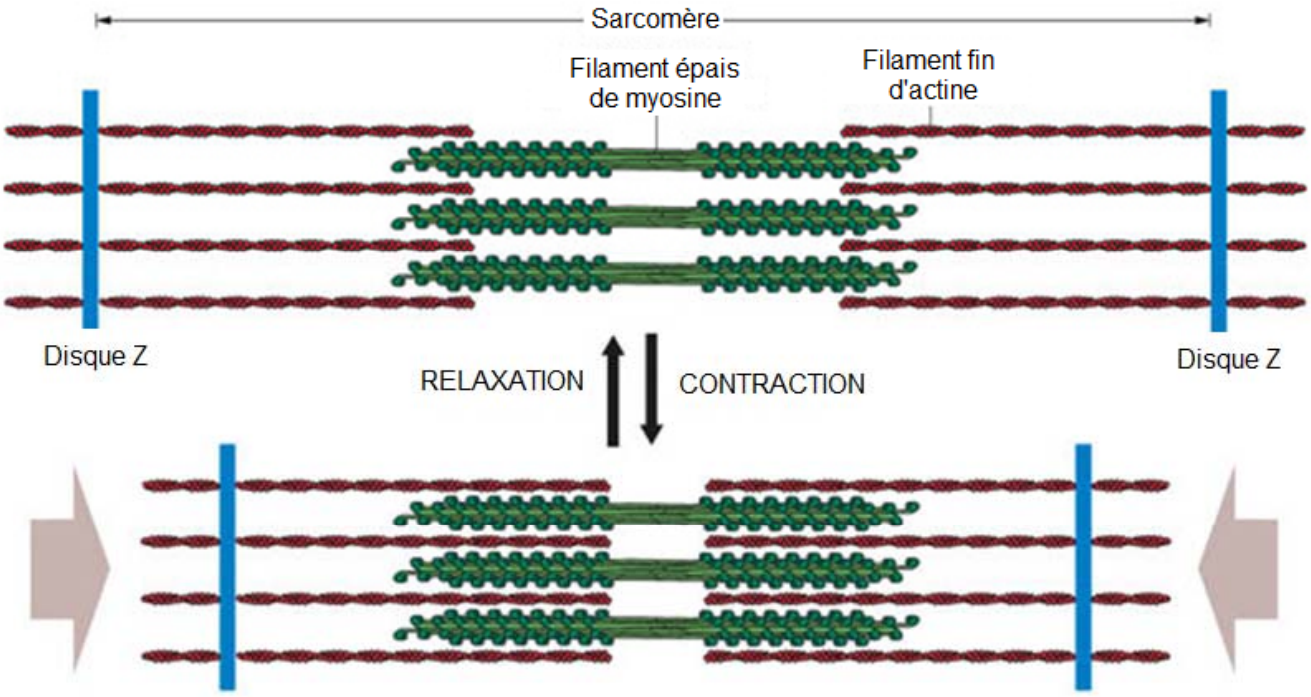

Figure 19: Contraction et relaxation musculaire selon la théorie « des filaments glissants » décrite par Huxley en 1957.

dihydropyridines (DHPR), sensible au potentiel d'action musculaire, et le récepteur à la ryanodine (RyR), canal de libération du Ca^{2+} au niveau de la membrane du RS (Lanner et al, 2010). L'ouverture de ces canaux conduit à un relargage du calcium du réticulum sarcoplasmique dans le sarcoplasme de la fibre musculaire (Baylor & Hollingworth, 2012).

- Le raccourcissement des sarcomères : suite à l'élévation de la concentration de calcium dans le sarcoplasme, les sites de fixation des ions Ca^{2+} de la troponine C sont rapidement saturés. Ceci induit un changement de la conformation de l'ensemble tropomyosine/troponines, exposant les sites de fixation spécifiques de la myosine présents sur le filament fin d'actine. La fonction ATPasique de la myosine est alors activée, induisant une hydrolyse de l'ATP en ADP+Pi, et les têtes de myosines vont alors se fixer à leurs sites spécifiques au niveau des filaments d'actine. Le Pi et l'ADP se détachent ensuite du complexe provoquant une flexion angulaire des têtes de myosines et donc le glissement des filaments fins d'actine sur les filaments épais de myosine. Ce mouvement est à l'origine de la contraction (Figure 20). La fixation d'une nouvelle molécule d'ATP sur les têtes globulaires de myosine induit la rupture du pont de liaison et le redressement des têtes de myosine (Lymn & Taylor, 1971). Si la concentration en Ca^{2+} est suffisamment élevée, le cycle se reproduit.

- Le relâchement du muscle : lorsque la concentration de Ca^{2+} cytoplasmique retourne à des valeurs de repos, le calcium se détache de la troponine C, entraînant un changement de conformation du complexe tropomyosine/troponines permettant à la tropomyosine de masquer à nouveau les sites actifs de l'actine (Gordon et al, 2000). Le Ca^{2+} est ensuite recapturé par le RS grâce à l'action d'une pompe calcique ATPasique, la pompe SERCA (Sarcoplasmique/Endoplasmique Reticulum Calcium-ATPase). Ce calcium va être ainsi séquestré et complexé à une protéine spécifique, la calséquestrine (Novak & Soukup, 2011).

4. Les différents types de fibres musculaires

En 1923, Needham décrivait deux types de muscle squelettique possédant chacun un type de fibres spécifiques (Needham, 1926):

- des muscles lents dits « rouges » ayant des fibres riches en myoglobine et en mitochondrie, caractérisés par un métabolisme oxydatif et impliqués dans une activité tonique. Ces fibres seront appelées fibres de type I.

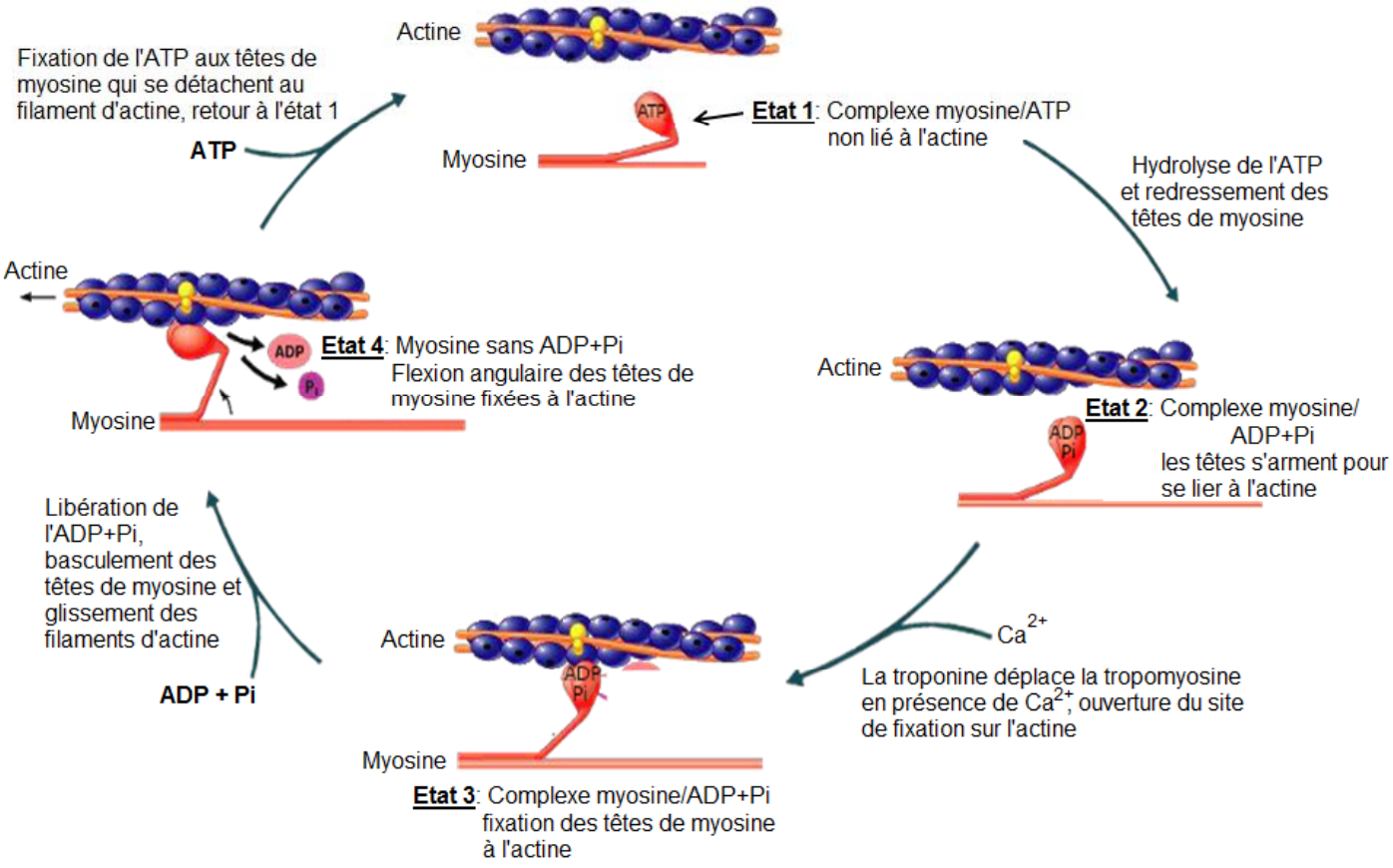

Figure 20: Cycle des interactions entre actine et myosine au cours de la contraction musculaire.

- des muscles rapides dits « blancs » ayant des fibres pauvres en myoglobine et en mitochondrie, caractérisés par un métabolisme glycolytique et impliqués dans une activité intense. Ces fibres seront nommées fibres de type II.

Dans les années 1970, la classification de ces types de fibres a évolué. L'analyse des différents types d'unités motrices et le marquage d'une enzyme impliquée dans le métabolisme oxydatif, la succinate déshydrogénase (SDH), au sein d'un muscle « blanc » de rat, a permis de mettre en évidence qu'il existait deux types de fibres de type II ayant un métabolisme différent (Close, 1967 ; Schiaffino et al, 1970). En effet, bien que ces fibres soient innervées par des unités motrices rapides (F), certaines ont un métabolisme plus oxydatifs que d'autres. C'est en 1970 que les travaux de Brooke et Kaiser ont véritablement permis de distinguer trois types de fibres musculaires via un marquage de l'activité ATPase (Brooke & Kaiser, 1970). Elles sont nommés I (dites « lentes oxydatives »), IIA (dites « rapides oxydatives et glycolytiques ») et IIB (dites « rapides glycolytiques »).

Il faut attendre les années 1990 et le développement de la technique d'immunohistologie permettant de marquer les différentes isoformes de chaînes lourdes de myosines (MHC), pour voir apparaître un nouveau type de fibres dans les muscles de rat : les fibres de type IIX (Larsson et al, 1991). Durant ces mêmes années, la présence de fibres hybrides exprimant simultanément plusieurs isoformes de MHCs a été mise en évidence. Ainsi, chez les rongeurs, il existe sept types de fibres dont la transition se réalise selon le schéma suivant : I ↔ I-IIA ↔ IIA ↔ IIA-IIX ↔ IIX ↔ IIX-IIB ↔ IIB (DeNardi et al, 1993).

Suite à ces nouvelles découvertes dans les muscles de rongeurs, la question des différents types de fibres musculaires chez l'Homme a été reconsidérée (Schiaffino, 2010 ; Schiaffino & Reggiani, 2011). Des analyses d'hybridation *in situ* et de PCR sur fibres isolées ont montré que les fibres classées IIB *via* le marquage ATPase chez l'Homme possédaient en réalité des isoformes de MHCs de type IIX (Ennion et al, 1995; Smerdu et al, 1994). Ainsi, chez l'Homme, la classification des différents types de fibres est la suivante :
I ↔ I-IIA ↔ IIA ↔ IIA-IIX ↔ IIX.
Toutes ces fibres ont des caractéristiques structurales, fonctionnelles et métaboliques différentes résumées dans le Tableau 2. La distribution de ces types de fibres est variable dans les muscles du corps humain. Les muscles squelettiques, et notamment les muscles de la jambe (ex, le muscle *vastus lateralis*), sont hétérogènes dans leur composition en fibres

Nomenclature	I	IIA	IIX
Caractéristiques fonctionnelles			
Unité motrice	Slow Fatiguable Resistant (S)	Fast Fatiguable Resistant (FR)	Fast Fatigable (FF)
Contraction	Lente	Rapide	Rapide
Résistance à la fatigue	+++	++	+
Caractéristiques structurales			
Couleur	Rouge	Rose	Blanche
Surface plaque motrice	+	+++	+++
Réticulum sarcoplasmique	+	+++	+++
Vascularisation	Elevée	Intermédiaire	Faible
Nombre de mitochondries	+++	++	+
Epaisseur du disque Z	+++	++	+
Richesse en collagène	+++	++	++
Caractéristiques métaboliques			
ATPase myofibrillaire	+	+++	+++
Teneur en glycogène	+	++	+++
Teneur en lipides	+++	++	+
Contenu en myoglobine	+++	++	+
Enzymes glycolytiques	+	++	+++
Enzymes mitonchondriales	+++	++	+

Tableau 2: Caractéristiques fonctionnelles, structurales et métaboliques des fibres musculaires chez l'homme.
D'après : Schiaffino, 2010 ; Schiaffino & Reggiani, 2011.

musculaires. Ceci leur permet une flexibilité dans l'utilisation d'un même muscle pour diverses tâches allant d'une activité continue peu intense (ex, posture), à des mouvements de contraction répétés (ex, locomotion), en passant par des contractions rapides et intenses (ex, saut ou coup de pied).

II. Métabolisme énergétique du muscle squelettique : substrats et voies métaboliques

1. Métabolisme des acides aminés

Plusieurs centaines d'acides aminés ont été identifiés, mais seule une vingtaine est utilisée comme source de synthèse protéique chez les eucaryotes supérieurs. Ces derniers sont nommés acides α-aminés naturels.

Le muscle squelettique est le principal réservoir d'acides aminés, stockés sous forme de protéines, ce qui représente une réserve très importante d'énergie et de nutriments utilisables par les tissus non musculaires en cas de besoin. En effet, en plus de leur rôle principal de substrat dans la synthèse protéique, les acides aminés peuvent être impliqués dans différentes voies métaboliques (précurseurs du cycle de Krebs, du cycle de l'urée, de la néoglucogenèse), participer à la synthèse de neuromédiateurs naturels (GABA, glutamate), ou encore assurer la fonction d'hormones (sérotonine, catécholamines), de molécules « signal » (arginine → NO, l'oxyde nitrique, aboutissant à une vasodilation de l'endothélium vasculaire) ou de nucléotides (glycine → purines, aspartate → pyrimidines) (Delgado, 2013; Deng et al, 2008 ; Szyperski, 1995).

En période post-prandiale (consécutive au repas), les acides aminés fournis par les protéines alimentaires affluent dans le plasma. Ils sont utilisés pour la synthèse des protéines corporelles qui vont alors être stockées : c'est l'anabolisme post-prandial, caractérisé par une balance azotée positive. Ainsi, 72% de l'azote ingéré au cours du repas se retrouve au niveau corporel, dont 31% dans le muscle au cours de cette phase (Bos et al, 2005). Au cours de la période post-absorptive, les protéines corporelles sont dégradées afin d'approvisionner les tissus en acides aminés ; il s'agit donc d'une période de catabolisme caractérisée par une balance azotée négative. Le catabolisme des 20 acides α-aminés aboutit à la formation de sept composés : le pyruvate, des intermédiaires du cycle de Krebs (oxaloacétate, α-

cétoglutarate, succinyl-CoA et furamate), l'acétyl-CoA et l'acétoacétyl-CoA (Figure 21) (Valerio et al, 2011). Les acides aminés servant de substrat pour la formation d'acétyl-CoA et d'acétoacétyl-CoA sont dits « cétogènes » puisqu'ils contribuent à la formation de corps cétoniques. En revanche, ceux utilisés pour la formation de pyruvate ou d'intermédiaires du cycle de Krebs sont appelés glucogéniques ou glucoformateurs. En effet, ils peuvent être convertis en phosphoénolpyruvate afin d'alimenter la voie de la néoglucogénèse (Stryer, 1995). Six acides aminés peuvent être métabolisés par le muscle squelettique au repos : les acides aminés à chaine ramifiée (leucine, isoleucine, valine), l'asparagine, l'acide aspartique, et l'acide glutamique (Wagenmakers, 1998).

2. Métabolisme glucidique

Le muscle squelettique est un organe majeur dans le maintien de l'homéostasie du glucose, substrat majeur et essentiel pour la production d'énergie permettant la contraction musculaire. Cependant, en plus de leur rôle énergétique, les glucides permettent également la synthèse de glycoprotéines, de macromolécules, de nucléotides, ou encore ils aident à l'épuration des produits insolubles et toxiques.

Suite à la prise alimentaire, l'élévation de la glycémie sanguine induit une sécrétion d'insuline par le pancréas. Au niveau de la cellule musculaire, l'insuline active les transporteurs spécifiques de glucose GLUT 4, entraînant une entrée accrue de glucose qui sera soit stocké sous forme de glycogène (glycogènèse), soit dégradé afin de produire de l'ATP (glycolyse). Ainsi, le maintien de la glycémie est dépendante de la sensibilité du muscle squelettique à l'action de l'insuline, le muscle est donc un organe insulino-dépendant (Sinacore & Gulve, 1993). Suite à une période de jeûne ou lors d'un effort physique, le muscle va pouvoir hydrolyser le glycogène stocké (glycogènolyse) pour produire du glucose-6-phosphate, premier intermédiaire de la glycolyse. Cependant, le muscle ne peut en aucun cas reverser du glucose dans le sang pour d'autres organes car il ne possède pas la glucose-6-phosphatase permettant le retour au glucose. De cette manière, tout le glucose entrant dans les muscles est strictement utilisé par les muscles.

Lors de la glycolyse, dont les étapes sont décrites figure 22, la transformation d'une molécule de glucose-6-phosphate en deux molécules de pyruvate conduit à la production de 4 ATP. Par la suite, en condition aérobie, ce pyruvate entre dans la mitochondrie où il sera

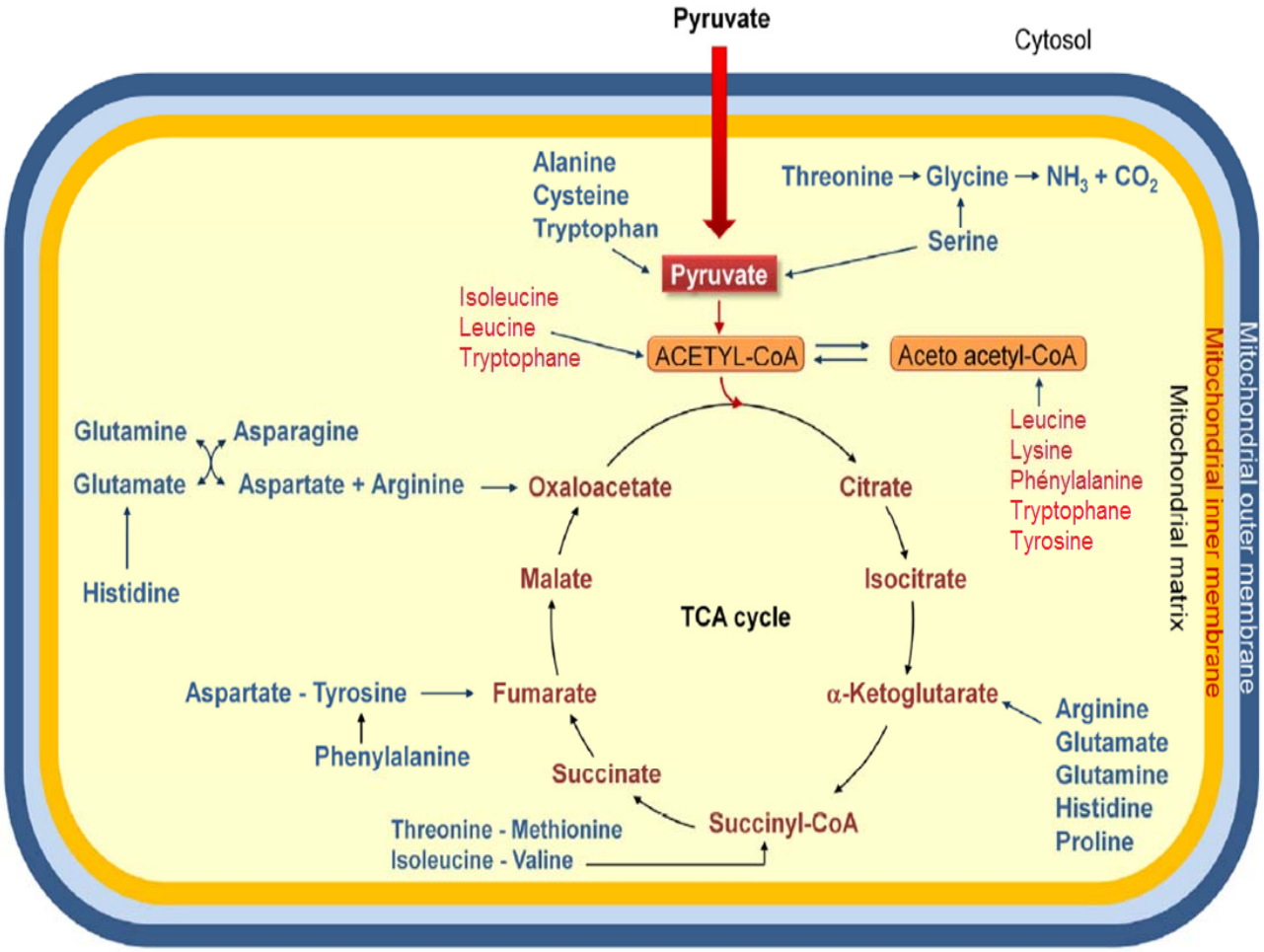

Figure 21: Formation de pyruvate, des intermédiaires du cycle de Krebs (oxaloacétate, α-cétoglutarate, succinyl-CoA et furamate), d'acétyl-CoA et d'acétoacétyl-CoA suite au catabolisme des acides aminés.
Certains de ces acides aminés sont dits « glucogéniques » (en bleu) et d'autres sont nommés « cétogènes » (en rouge).
D'après: **Valerio** et al., 2011.

transformé en acétyl-coenzyme A qui sera dégradé par le cycle de Krebs (Figure 21), conduisant à la formation de 32 ATP. En condition anaérobie, le pyruvate formera de l'acide lactique (lactate) qui sera envoyé continuellement vers le foie permettant ainsi une production rapide d'énergie (2 ATP) lors d'un effort important (McArdle et al, 2007).

Comme décrit précédemment, un même muscle peut posséder à la fois des fibres musculaires oxydatives, riches en mitochondrie, et des fibres musculaires glycolytiques, très pauvres en mitochondrie. Ainsi, l'utilisation de ces différents systèmes énergétiques sera fonction du type d'activité physique engagé. Le système oxydatif aérobie sera utilisé pour de longues périodes d'activité de faible intensité, tandis que le système glycolytique anaérobie sera utilisé lors d'activités courtes mais de forte intensité (Figure 22) (Spriet et al, 2000 ; Wells et al, 2009).

3. Métabolisme lipidique

En situation de jeûne, l'oxydation lipidique devient majoritaire par rapport à l'oxydation glucidique dans le muscle squelettique (Dagenais et al, 1976). Ainsi, les acides gras (AG) circulants libérés par le tissu adipeux et captés par le muscle, et les AG issus de l'hydrolyse des triglycérides musculaires constituent une source d'énergie importante.

Les AG libres circulants sont transportés par l'albumine. Ils entrent dans la cellule musculaire par diffusion passive ou facilitée par la protéine FAT/CD36 (Fatty Acid Transporter) et la FABPpm (plasma membrane-associated Fatty Acid Binding Protein). Néanmoins, l'entrée des AG libres peut se faire également par un mécanisme de transport facilité grâce à une famille de FATP1-6 (« Fatty Acid Transport Proteins ») (Nickerson et al, 2009). Une fois entrés dans la cellule, les acides gras sont ensuite transportés à travers le cytosol par FABPc (cytosolic Fatty Acid Binding Protein) pour être stockés sous forme de triglycérides (TG) ou oxydés au niveau mitochondrial selon les besoins.

Le muscle squelettique est en effet capable de stocker des TG qui seront directement disponibles pour être dégradés en cas de besoins énergétiques, notamment lors d'une activité musculaire prolongée. Ce stockage est réalisé dans des organites spécifiques nommées « gouttelettes lipidiques » ou LDs (Lipid Droplets). Ces structures contiennent un noyau de lipides neutres, principalement des TG et des esters de cholestérol, entouré par une monocouche de phospholipides et de protéines associées (Tauchi-Sato et al, 2002 ; Guo et

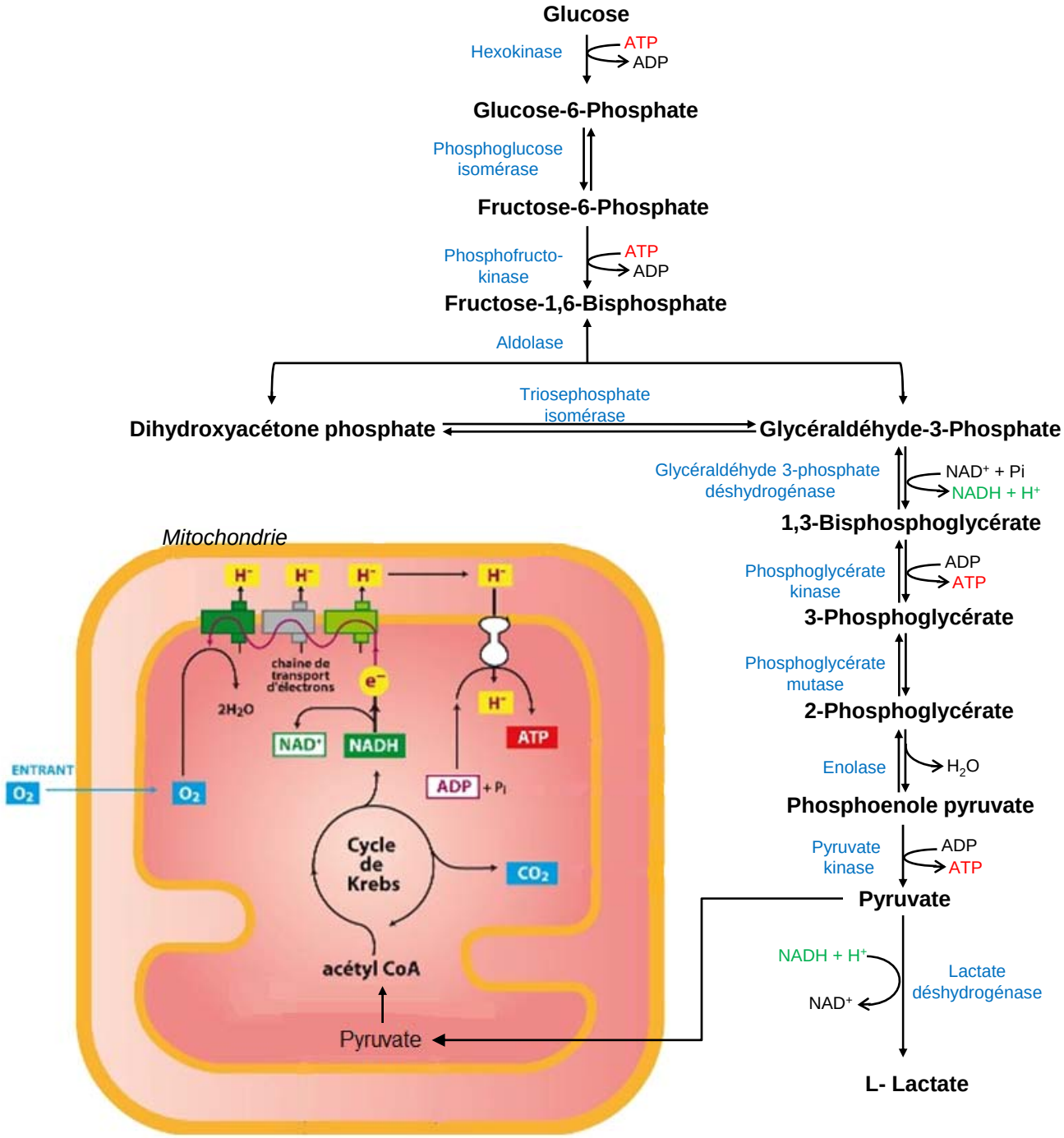

Figure 22: Représentation schématique des différentes étapes de la glycolyse, aboutissant à la formation de pyruvate. En milieu aérobie, ce pyruvate entre dans la mitochondrie où il servira de substrat pour le cycle de Krebs. En milieu anaérobie, le pyruvate est convertie en lactate, qui sera envoyé vers le foie.

al, 2009) (Figure 23). Jusqu'à récemment, ces LDs apparaissaient comme un lieu de stockage inerte, mais l'évolution des techniques d'imagerie et de biologie moléculaire ont permis de faire évoluer la définition de la gouttelette lipidique, devenant une véritablement organelle dynamique et régulée. En effet, l'identification de protéines spécifiques associées aux LDs représente la première étape vers la reconnaissance des gouttelettes comme étant des organelles intracellulaires (Le Lay & Dugail, 2009; Martin & Parton, 2006). Les protéines associées aux LDs les plus abondantes appartiennent à la famille PAT dont les membres fondateurs sont la périlipine, l'ADRP (Adipose Differentiation-Related Protein, ou adipophiline) et la TIP47 (Tail-Interacting Protein of 47 kDa) (Lu et al, 2001). Ces protéines ont été décrites comme ayant un rôle dans la signalisation cellulaire, notamment dans la gestion du stock de lipides et dans la régulation de l'activité des lipases (Watt & Hoy, 2012). Actuellement, trois enzymes sont connues pour avoir une fonction dans la régulation de la lipolyse : ATGL (Adipose Triglyceride Lipase), HSL (Hormone-Sensitive lipase) et MGL (MonoGlyceride Lipase) (Chang & Chan, 2007 ; Zechner et al, 2009). Ainsi, les TG musculaires sont dégradés par ces lipases en glycérol et en acides gras qui seront directement oxydés.

L'oxydation des acides gras est mitochondriale, elle est nommée β-oxydation, et aboutit à la production d'équivalents réducteurs (FADH + H+, NADH + H+) pouvant alimenter la chaîne respiratoire et à la production d'acétyl-CoA qui pourra entrer dans le cycle de Krebs afin de fournir de l'énergie (Wakil, 2012). Dans le muscle squelettique, cette étape de β-oxydation est facilitée par la localisation des LDs à proximité des mitochondries (Shaw et al, 2008). Une représentation schématique de la β-oxydation des lipides dans la mitochondrie est proposée dans la figure 24.

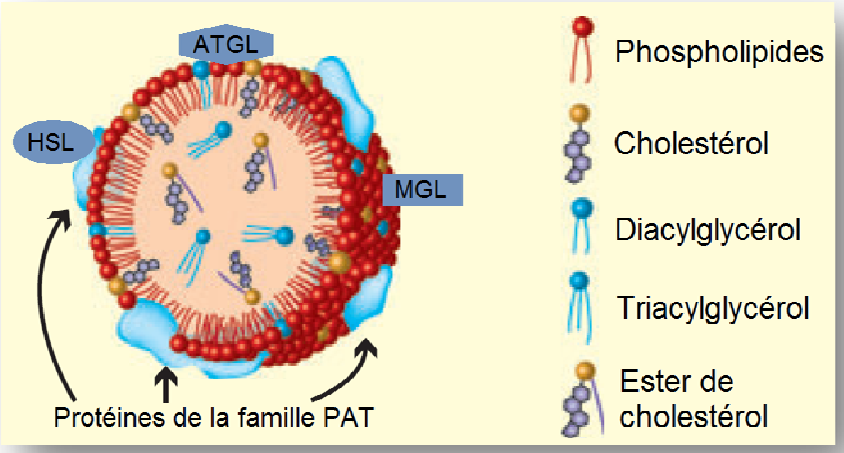

Figure 23: Composition d'une gouttelette lipidique et de ses protéines associées: les protéines de la famille PAT (Périlipine, ADRP, TIP47) et les lipases (HSL, ATGL et MGL).
D'après: **Guo** et al, 2009; **Lu** et al, 2001.

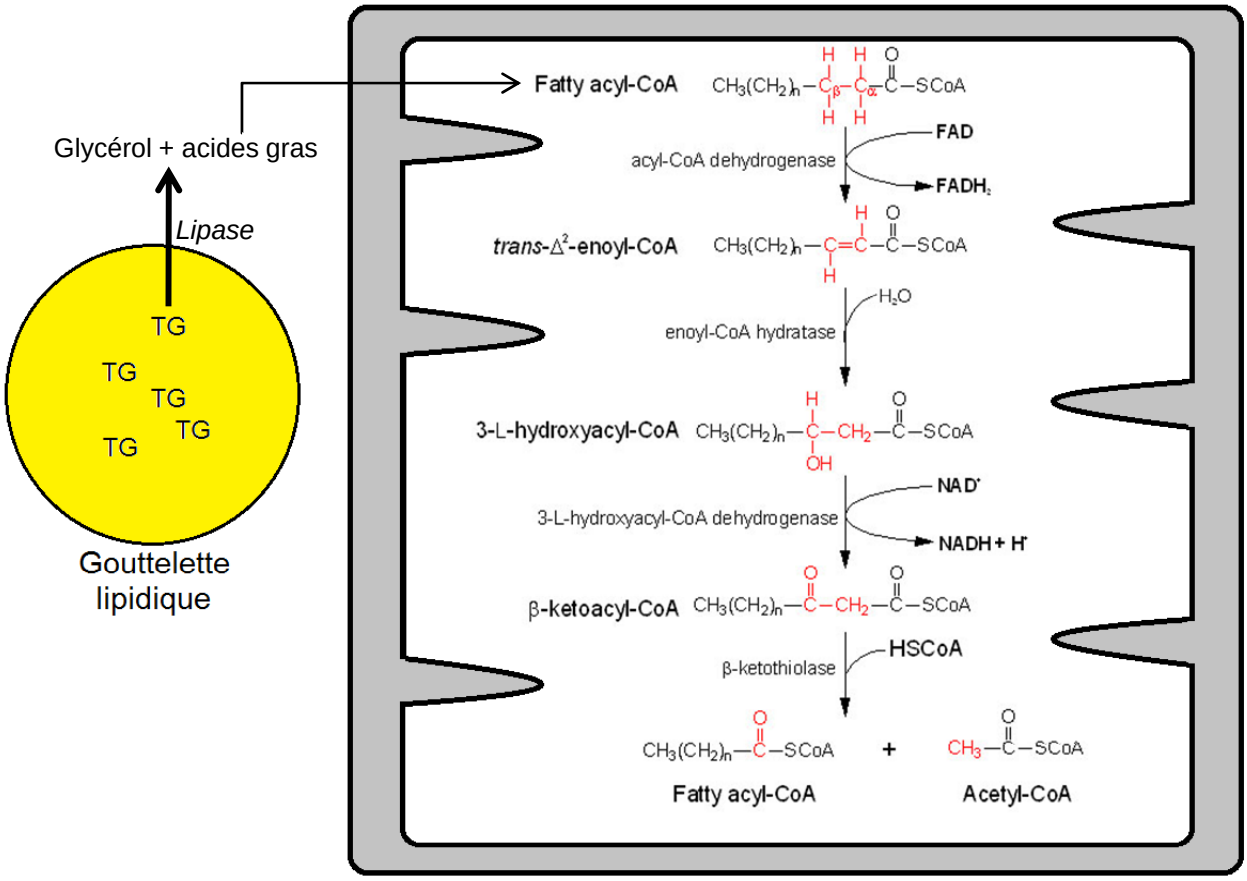

Figure 24: Représentation schématique des étapes de la β-oxydation des acides gras dans la mitochondrie.
D'après: **Wakil**, 2012

Chapitre III : Vieillissement musculaire

I. Les définitions de la sarcopénie

1. Introduction du terme « sarcopénie » et prévalence

Comme le décrit le Chapitre I, le vieillissement est caractérisé par une détérioration de nombreuses fonctions physiologiques, qui seules ou associées, sont à la base de la diminution des performances motrices et contribuent fortement à la perte d'autonomie des personnes âgées. La fonte musculaire constitue un élément critique, puisqu'elle est tenue pour responsable d'une part importante des incapacités motrices survenant avec l'âge. La notion de sarcopénie est apparue au cours des années 1990 pour qualifier ce phénomène, ce terme dérivant des mots grecs *sarx* (chair) et *penia* (pauvreté) (Rosenberg, 1997). La sarcopénie, définie comme étant une perte de masse et de fonction du muscle squelettique liée à l'âge, est à l'origine d'une détérioration générale de l'état physique. A partir de 50 ans, on estime que l'Homme subit une perte d'environ 1 à 2 % de masse musculaire par an (Buford et al, 2010), menant à une réduction d'environ 50 % de la masse musculaire chez les personnes âgées de 80 ans et plus (Bijlsma et al, 2012; Faulkner et al, 2007 ; Lexell et al, 1988). Ceci se traduit par une augmentation du risque de chutes, une incapacité progressive à effectuer des activités basiques de la vie quotidienne, une perte d'autonomie, et conduit finalement à une réduction de l'espérance de vie (Delmonico et al, 2007; Goodpaster et al, 2006).

La sarcopénie est prévalente chez la personne âgée, et une diminution de 10,5 % de sa prévalence conduirait à une réduction importante des coûts de santé publique. Aux Etats Unis, la réduction de ces coûts a été estimée à environ 1,1 milliard de dollars par an (Janssen et al, 2004).

2. Une définition controversée

La prévalence et la mesure de l'impact de la sarcopénie dépend crucialement de la manière dont on la définit. En 1998, Baumgartner suggère une définition basée sur la masse maigre appendiculaire (kg) divisée par la taille (m) au carré, on parle ainsi de masse

musculaire absolue (Baumgartner et al, 1998). D'autres chercheurs s'appuient sur l'utilisation de la masse musculaire relative, correspondant à la masse maigre appendiculaire divisée par la masse corporelle totale et multipliée par 100 (Janssen et al, 2002). Ces deux index doivent être inférieurs à au moins deux écarts-type par rapport à celui d'une population de référence plus jeune pour diagnostiquer la sarcopénie. Cependant, le fait de prendre en compte seulement la masse musculaire pour le diagnostic de la sarcopénie, et non un paramètre fonctionnel est également critiquable. La mesure de la force musculaire, ou bien d'une performance physique, apparaissent en effet être des paramètres essentiels pour ce diagnostic. Dans ce contexte, plusieurs groupes venant des Etats-Unis et de l'Europe se sont attachés à mieux définir la sarcopénie ainsi que ses critères de diagnostic (Tableau 3) (Cooper et al, 2013). Ces différentes approches ont en commun un intérêt croissant pour l'utilisation potentielle de tests de force musculaire simples (ex, force du poigné), ou de performances physiques (ex, vitesse de marche) afin d'identifier des groupes de patients pouvant bénéficier d'interventions ciblées (Cooper et al, 2010).

3. Un diagnostic difficile

Toutes ces divergences rendent impossible la comparaison des études menées sur la sarcopénie dû à la très faible concordance entre les critères de diagnostic utilisés. Récemment, une étude a mis en évidence que la prévalence de la sarcopénie variait en fonction des critères de diagnostic, mais aussi en fonction de la population de référence utilisée (Bijlsma et al, 2013a). Cette étude a montré que, pour une même cohorte de personnes âgées, la prévalence de la sarcopénie fluctue entre 0 et 45,2 % en fonction des critères de diagnostics utilisés.

Définir le critère de diagnostic le plus précis afin d'étudier la sarcopénie reste actuellement difficile. Cependant, des travaux ont permis d'apporter des indications intéressantes quant à l'association de certains critères de diagnostic avec des altérations physiologiques liées à l'âge. Il a ainsi été mis en évidence que la masse musculaire relative était associée à la performance physique et à la résistance à l'insuline, contrairement à la masse musculaire absolue (Bijlsma et al, 2013b ; Bijlsma et al, 2013c). Ces différences entre masse musculaire absolue et relative peuvent être expliquées par le rôle de la masse grasse. Newman *et al*. ont démontré que la correction par la taille uniquement mène à une surestimation de la sarcopénie chez les individus en insuffisance pondérale et à une sous-estimation de la sarcopénie chez les individus obèses (Newman et al, 2003). Ceci laisse

Groupe d'étude	Définition	Critères de diagnostic
ESPEN Special Interest Groups (1)	« La sarcopénie est caractérisée par la perte de masse et de force musculaire. Bien qu'elle touche principalement les personnes âgées, son développement peut être associé avec d'autres conditions qui ne sont pas exclusivement observées lors du vieillissement, comme l'inactivité, la malnutrition ou la cachexie. De la même façon que l'ostéopénie, la sarcopénie peut également être observée chez des personnes atteintes de maladies inflammatoires. »	**1.** Masse musculaire faible, ex, pourcentage de masse musculaire > à 2 SD en-dessous de la moyenne observée chez des individus âgés de 18 à 39 ans et appartenant à la cohorte NHANES III. **2.** Vitesse de marche < à 0,8 m/s durant un test de 4 minutes ou une performance réduite dans tous les tests fonctionnels utilisés pour une évaluation gériatrique complète
European Working Group on Sarcopenia in Older People (2)	« La sarcopénie est un syndrome caractérisé par la perte progressive et généralisée de la masse et de la force musculaire squelettique avec un risque d'effets indésirables tels qu'un handicap physique, une mauvaise qualité de vie et le décès. » Ce syndrome est appelé « sarcopénie primaire » lorsque la cause est uniquement le vieillissement, et « sarcopénie secondaire » quand une maladie, une inactivité ou une malnutrition contribue à son apparition.	**1.** Faible masse musculaire **2.** Faible force musculaire (ex, force de préhension) **3.** Faible performance physique (ex, vitesse de marche) Le critère 1 avec le critère 2 ou 3 doivent être < à 2 SD par rapport à la moyenne d'une population de référence composée de personnes jeunes et saines.
International Working Group on Sarcopenia (3)	« La sarcopénie est définie comme étant une perte de masse et de fonction du muscle squelettique lié à l'âge avancé. Les causes de la sarcopénie sont multifactorielles et peuvent inclure l'inactivité, une altération de la fonction endocrinienne, une maladie chronique, l'inflammation, l'insulino-résistance, ainsi que des déficiences nutritionnelles. Bien que la cachexie puisse être une composante de la sarcopénie, ces deux états sont différents. »	**1.** Vitesse de marche < à 1 m/s **2.** Faible masse musculaire mesurée objectivement, ex, mesure de la masse appendiculaire par rapport à la taille au carré dont les valeurs doivent être ≤ à 7,23 kg/m^2 pour les hommes et ≤ à 5,67 kg/m^2 pour les femmes.
Society of Sarcopenia, Cachexia and Wasting Disorders (4)	« La sarcopénie avec une mobilité réduite est une condition spécifique montrant une nette perte de masse musculaire et étant une cible claire d'interventions. Ainsi, elle diffère de la notion générale de fragilité. » « La limitation de la mobilité ne doit pas être clairement attribuable à une maladie spécifique ou à des troubles du système nerveux périphérique et central, des démences ou une cachexie. »	**1.** Vitesse de marche ≤ à 1 m/s ou une distance de marche < à 400 m au cours d'une durée de marche de 6 minutes. **2.** La masse maigre appendiculaire doit être < à 2 SD par rapport à la moyenne de personnes saines âgées de 20 à 30 ans appartenant au même groupe ethnique.

Tableau 3: Définitions et critères de diagnostic de la sarcopénie suggérés par plusieurs groupes provenant des Etats Unis et de l'Europe.
D'après: Muscariti et al, 2010 (1), Cruz-Jentoft et al, 2010 (2), Fielding et al, 2011 (3) et Morley et al, 2011 (4).

penser que le calcul de la masse musculaire relative conduit à une meilleure estimation de la sarcopénie chez les personnes ayant un indice de masse corporelle « anormal ».

4. Vieillissement musculaire ou sarcopénie?

Le vieillissement musculaire est caractérisé par une perte de masse et de force musculaire. Cependant, l'association de ces deux paramètres pour définir la sarcopénie ne fait pas l'unanimité et certains auteurs préfèrent qualifier la perte de force musculaire liée à l'âge de « dynapénie » (Clark & Manini, 2008 ; Mitchell et al, 2012). En effet, les partisans de la conception classique de la sarcopénie considèrent que les altérations de la force et de l'endurance musculaire sont uniquement imputables à l'atrophie musculaire. Néanmoins, cette conception omet la diminution avec l'âge de la force spécifique (c'est-à-dire la force développée par rapport à la surface du muscle) qui souligne des altérations contractiles avec l'âge totalement indépendantes de l'atrophie musculaire : altération du contrôle neuronal, troubles cognitifs, perturbations du système cardiovasculaire et articulaire (Doherty, 2003).

Ainsi, de meilleures connaissances sur les conséquences cliniques de la sarcopénie et l'établissement de seuils de diagnostics aideraient à régler définitivement le débat. Etant donné le manque d'une définition claire et précise, et afin de s'affranchir des divergences autour de la sarcopénie dans la littérature, au cours de cette thèse, nous parlerons de « vieillissement musculaire » et non de « sarcopénie ».

II. Conséquence du vieillissement sur la structure et la fonction du muscle squelettique

1. Modification de la composition et de la structure des fibres musculaires

a- Controverse sur la proportion des différents types de fibres musculaires

Le vieillissement conduit à d'importants changements de la structure et de la composition des fibres musculaires squelettiques. Au début des années 1980, Jan Lexell a décrit ces changements suite à des analyses post-mortem du muscle *vastus lateralis* de personnes jeunes (30 ans) et âgées (72 ans). Ces travaux ont ainsi pu montrer que le

vieillissement était associé à une diminution de l'aire occupée par le muscle (- 18 %) et du nombre total de fibres musculaires (- 25 %) (Lexell et al, 1983 ; Lexell et al, 1988). Cependant, dans le muscle *vastus lateralis*, aucune différence significative dans la proportion des différents types de fibres n'a été observée. Ce résultat reste néanmoins controversé dans la littérature. En effet, alors que certains auteurs n'observent pas ou peu de changement dans la proportion des différents types de fibres (D'Antona et al, 2003 ; Klitgaard et al, 1990 ; Kosek et al, 2006; Lexell & Taylor, 1991 ; Lexell et al, 1988), d'autres montrent une augmentation significative des fibres de type I (Dreyer et al, 2006 ; Poggi et al, 1987 ; Verdijk et al, 2007), ou une diminution significative des fibres de type II (Dreyer et al, 2006 ; Nilwik et al, 2013; Verdijk et al, 2007).

Ces différences peuvent être expliquées par la grande diversité inter-individuelle mais aussi par la variabilité de la composition du muscle en fonction de la région biopsiée (Lexell & Taylor, 1991). De plus, dans leur revue, Narici et Maffulli expliquent que lors du vieillissement, une perte à la fois des fibres de type I et des fibres de type II survient mais à des temps différents (Narici & Maffulli, 2010). Alors qu'il y aurait une perte préférentielle des fibres de type II jusqu'à 70-80 ans, une chute du nombre de fibres de type I se produit par la suite, créant une nouvelle « balance ». Ceci suggère alors que la proportion des fibres de type I et de type II devient similaire chez les personnes âgées de plus de 85 ans (Andersen, 2003 ; Narici & Maffulli, 2010).

b- Unités motrices et fibres hybrides

Le vieillissement est associé à une diminution du nombre de motoneurones α (Doherty & Brown, 1993), ce qui a pour conséquence une perte d'unités motrices dans les muscles des personnes âgées (Campbell et al, 1973 ; McNeil et al, 2005; Vandervoort, 2002). Campbell a rapporté que le nombre d'unités motrices reste relativement constant jusqu'à l'âge de 60 ans, puis chute rapidement à un taux de 3 % par an, ce qui correspond à une perte de 60 % à l'âge de 80 ans (Campbell et al, 1973). Cependant, des fibres musculaires dites « orphelines » sont souvent ré-innervées par une des unités motrices existantes *via* des bourgeonnements collatéraux (Roos et al, 1997). Outre la réduction du nombre, lors du vieillissement, chaque motoneurone innerve plus de fibres musculaires que dans un muscle jeune, les unités motrices deviennent ainsi beaucoup plus large (Stalberg et al, 1989). Toutes ces altérations dans le remodelage des unités motrices, c'est-à-dire dans les processus de dénervation/réinnervation,

semblent conduire à un regroupement des fibres musculaires en fonction de leur type (Lexell & Downham, 1991). Cette dénervation semble affecter préférentiellement les fibres de type II, et elle est suivie d'une ré-innervation *via* le bourgeonnement collatéral d'unités motrices voisines normalement associées à des fibres de type I (Andersen, 2003; Roos et al, 1997 ; Vandervoort, 2002). Ce phénomène peut ainsi conduire à des déficiences dans le contrôle moteur et dans la production de la force.

La dénervation/réinnervation des fibres de type II dans les muscles squelettiques âgés, a également été impliquée dans l'augmentation de la proportion des fibres hybrides co-exprimant deux ou trois types de MHC (Andersen et al, 1999; Roos et al, 1997). Dans des études réalisées à partir de biopsies du muscle *vastus lateralis* de personnes âgées (73 ans) et très âgées (88 ans), le nombre de fibres co-exprimant deux types de MHC, en particulier les fibres de type I-IIA, serait fortement élevé par rapport au nombre de fibres hybrides présentes dans des muscles de personnes jeunes (Andersen et al, 1999 ; D'Antona et al, 2003). Cependant, ce résultat est assez controversé dans la littérature (Larsson et al, 1997), et il semble être dépendant de la technique utilisée pour effectuer le typage contractile du muscle. En effet, pour un même échantillon, les techniques d'histochimie (marquage de l'activité ATPase), d'immunohistologie (marquage des différentes isoformes de MHC) et d'électrophorèse sur fibres isolées n'indiquent pas la même proportion de fibres hybrides (Andersen, 2003; Pette et al, 1999 ; Serrano et al, 2001). Ceci pourrait être expliqué par une répartition des différentes isoformes de MHC pouvant être soit uniforme, soit hétérogène tout au long de la fibre musculaire (Staron & Pette, 1987). Dans sa revue, Andersen explique que chaque noyau d'une fibre musculaire contrôle un domaine où les ARNm transcrits sont traduits en protéines et incorporés localement dans ce domaine (Andersen, 2003). En condition « normale », une coordination stricte entre les noyaux permet d'assurer qu'une fibre de type I reste une fibre de type I et qu'une fibre de type II reste une fibre de type II. Or, si cette coordination est altérée, un noyau pourra probablement produire et incorporer dans son domaine nucléaire, une isoforme de MHC qui différera de celles présentes dans le reste de la fibre (Andersen, 2003). Ainsi, il devient assez facile de comprendre pourquoi les techniques d'histochimie et d'immunohistologie, réalisées sur des coupes transversales d'environ 10 µm d'épaisseur, ne permettent pas une quantification précise des fibres hybrides, contrairement à la technique d'électrophorèse sur fibres isolées (Figure 25).

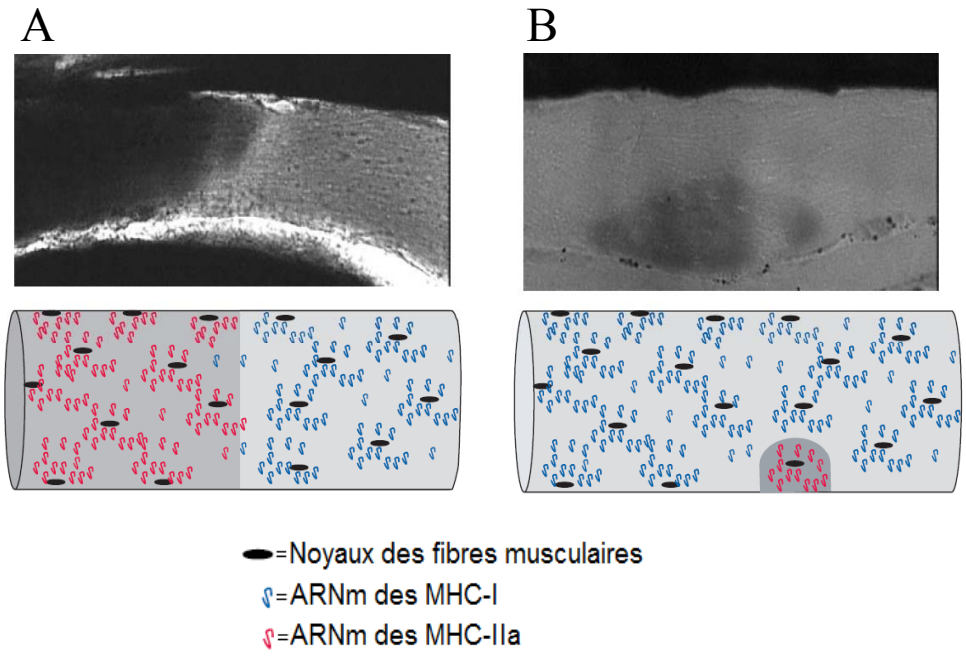

Figure 25: Marquage ATPase de fibres musculaires longitudinales provenant de personnes âgées (> 85 ans). Les schémas ci-dessus montrent la répartition de l'expression des ARNm des isoformes I et II des MHC. **A:** La fibre musculaire subit un changement de phénotype qui se propage le long de la fibre. **B:** Exemple de marquage montrant un changement de l'expression des isoformes de MHC au niveau d'un domaine nucléaire.
D'après: **Andersen** et al, 2003

Ainsi, il est tout à fait envisageable que chez les personnes âgées, la coordination entre les noyaux soit altérée, conduisant à une augmentation de la proportion de fibres hybrides, uniquement observée via la technique d'électrophorèse sur fibres isolées (Andersen, 2003; Andersen et al, 1999 ; D'Antona et al, 2003).

c- Atrophie des fibres de type II

Contrairement à la proportion des fibres musculaires, un consensus est admis dans la littérature concernant l'aire des fibres musculaires. En effet, de nombreuses études ont mis en évidence une diminution significative de l'aire des fibres de type II lors du vieillissement (Dreyer et al, 2006 ; Hakkinen et al, 2001 ; Klitgaard et al, 1990 ; Larsson et al, 1997 ; Lexell & Taylor, 1991 ; Lexell et al, 1988 ; Verdijk et al, 2007), alors que l'aire des fibres de type I est peu affectée (Dreyer et al, 2006 ; Klitgaard et al, 1990 ; Lexell & Taylor, 1991 ; Verdijk et al, 2007) (Figue 26). Les conséquences de cette atrophie spécifique des fibres de type II restent encore à approfondir. Récemment, une étude a montré que la perte de masse musculaire squelettique, observée chez les personnes âgées, semble être attribuée à une atrophie de ces fibres (Nilwik et al, 2013). En parallèle, les auteurs décrivent également que l'augmentation de la masse musculaire, observée chez les personnes âgées soumises à un entraînement physique, est la conséquence d'une hypertrophie des fibres de type II (Verdijk et al, 2009). Ces observations mettent alors en évidence l'importance et le rôle essentiel de ces fibres dans le vieillissement musculaire.

d- Déformation des fibres musculaires

Deux indices ont été utilisé dans la littérature pour quantifier la déformation des fibres musculaires : la circularité, qui est égale à 4π x aire / périmètre^2, et le facteur de forme SF (en anglais, Shape Factor), qui est la réciproque inverse de la circularité et qui est égal au périmètre^2 / 4π x aire (Bruusgaard et al, 2006 ; Kirkeby & Garbarsch, 2000 ; Verdijk et al, 2007). Un SF égale à 1 correspond à une fibre parfaitement ronde, plus ce facteur est augmenté, plus la fibre sera déformée. Un changement de la forme des fibres musculaires est très souvent associé à des processus neuropathiques comme une dénervation chronique. Ainsi, chez les personnes âgées, suite à une perte de motoneurones, les fibres deviennent atrophiques et prennent une forme dite « angulaire ». Bien que peu d'études se soient intéressées à l'apparition ces fibres angulaires lors du vieillissement, il semble que les fibres de type II

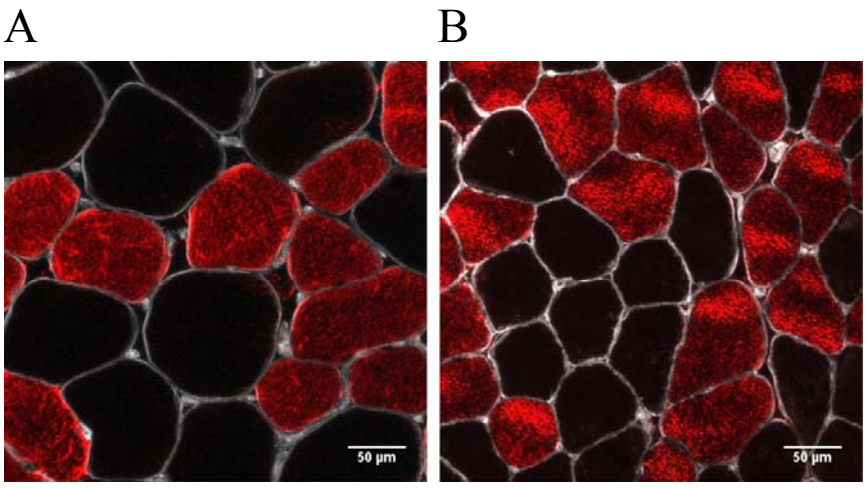

Figure 26: Marquages immunohistologiques de coupes transversales de fibres musculaires provenant de biopsies du muscle *vastus lateralis* de personnes jeunes (**A**) et âgées (**B**). Les marquages immunohistologiques des MHC-I (rouge) et de la laminine (gris) permettent de mettre en évidence la diminution de l'aire des fibres de type II lors du vieillissement.
D'après: **Nilwik** et al, 2013

soient plus affectées par ces changements que les fibres de type I (Figure 27) (Andersen, 2003; Kirkeby & Garbarsch, 2000).

2. Cellules satellites et vieillissement

Lors du vieillissement, l'efficacité de potentiel régénératif musculaire diminue, suggérant une altération de la fonction des cellules satellites. Le rôle et la contribution au phénotype du muscle âgé de ces cellules souches a récemment fait l'objet de nombreuses études. Bien que le nombre de cellules satellites présents dans des muscles de souris âgées soit encore discuté (Brack & Rando, 2007; Conboy et al, 2003 ; Gibson & Schultz, 1983 ; Shefer et al, 2006), chez l'Homme, un déclin dans le nombre de cellules exprimant des marqueurs de cellules satellites ou dans la position de ces cellules est fréquemment observé (Kadi et al, 2004 ; Renault et al, 2002 ; Sajko et al, 2004). De plus, cette diminution semble être plus marquée pour les fibres de type II que pour celles de type I. En effet, des études ont montré une diminution d'environ 44 % du contenu en cellules satellites pour les fibres de type II par rapport aux fibres de type I dans des muscles de personnes âgées, alors que ce contenu ne diffère pas entre ces deux types de fibres dans les muscles de personnes jeunes (Shefer et al, 2006 ; Verdijk et al, 2009; Verdijk et al, 2007 ; Verney et al, 2008). De plus, les travaux de Verdijk et al, en 2007, ont également mis en évidence une diminution significative du pourcentage de cellules satellites pour les fibres de type II dans le muscle âgé par rapport à un muscle jeune (Verdijk et al, 2007). Ainsi, la réduction du contenu de ces cellules pourrait représenter un facteur important dans l'atrophie de ce type de fibre observée lors du vieillissement.

Bien que le nombre de cellules satellites semble diminuer lors du vieillissement, les niveaux d'expression de certains facteurs de régulation musculaire, appelés MRFs (Muscle Regulatory Factors), comme MyoD, myf-5 ou la myogénine, apparaissent surexprimés dans les muscles âgés (Edstrom & Ulfhake, 2005 ; Kim et al, 2005 ; Kosek et al, 2006; Musaro et al, 1995). Dans les muscles de rongeurs, la magnitude de surexpression des ARNm des MRFs semble être proportionnelle au degré de la sarcopenie (Edstrom & Ulfhake, 2005). Il est alors envisageable que ces changements opèrent afin de compenser la perte des cellules satellites lors du vieillissement. Cependant, même si la sarcopénie est associée à un déclin du nombre de ces cellules, les cellules satellites résidentes restantes doivent être en nombre suffisant pour l'activation et le maintien d'un mécanisme de régénération convenable (Carlson

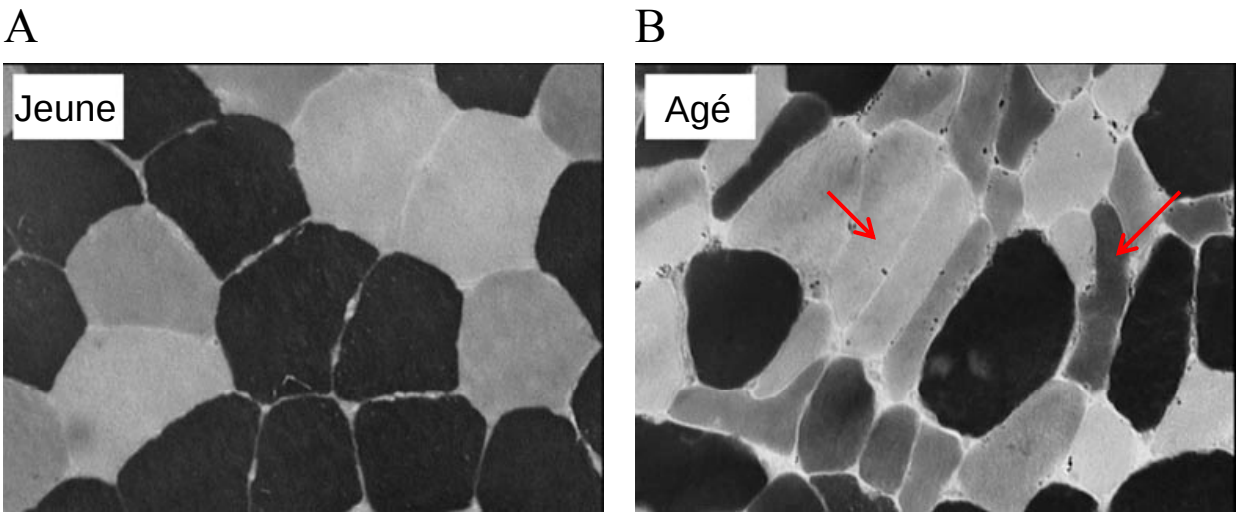

Figure 27: Marquages ATPase de coupes transversales de fibres musculaires provenant de biopsies du muscle *vastus lateralis* de personnes jeunes (**A**) et âgées (**B**). Les flèches montrent la déformation des fibres musculaires associée au vieillissement.
D'après: **Andersen** et al, 2003

et al, 2001). En effet, il a été démontré que les cellules satellites provenant de muscles de souris âgées maintiennent leur potentiel de réponse aux signaux, subissent une différenciation, fusionnent avec les myotubes et produisent des cellules de réserve tout au long de leur vie (Alsharidah et al, 2013; Collins et al, 2007 ; Shavlakadze et al, 2010 ; Shefer et al, 2006). Ceci suggère ainsi que l'altération de la régénération musculaire peut être due à un environnement vieillissant et pas nécessairement à des changements intrinsèques des cellules satellites. Des expériences de parasymbiose chez l'animal ont montré que la mise en relation de la circulation d'un organisme jeune avec celui d'un organisme âgé « rajeunissait » l'organisme vieillissant (Conboy et al, 2005). Ces résultats montrent l'importance de l'environnement dans lequel se trouvent les cellules satellites, qui est créée par des facteurs circulants, mais aussi par des facteurs sécrétés localement *via* les cellules environnantes et par le contexte inflammatoire présent lors des étapes précoces de la régénération musculaire (Bencze et al, 2012).

3. Une vascularisation perturbée

Le vieillissement est associé à une diminution du flux sanguin de 20 à 30 % dans les muscles squelettiques, lors de la contraction musculaire, et plus particulièrement dans les muscles de la jambe (Dinenno et al, 2001 ; Groot et al, 2013; Newcomer et al, 2005). Cette diminution peut en partie être expliquée par des altérations du réseau de capillaires irriguant les muscles. En effet, ce réseau joue un rôle majeur puisqu'il apporte l'oxygène et les substrats nécessaires au fonctionnement musculaire. Lors du vieillissement, plusieurs études ont montré une diminution de la densité de ces capillaires (Coggan et al, 1992 ; Frontera et al, 2000; Proctor et al, 1995), ainsi que des variations du nombre de capillaires en fonction du type de fibres. En effet, le nombre de capillaires entourant les fibres de type II semble diminué chez les personnes âgées, alors que les fibres de type I ne sont pas affectées, que ce soit chez l'homme ou chez la femme (Croley et al, 2005 ; Ryan et al, 2006). A l'état basal, le maintien de la capillarisation musculaire est permise par de nombreux facteurs angiogéniques, et plus particulièrement par le facteur de croissance de l'endothélium vasculaire VEGF (en anglais, Vascular Endothelium Growth Factor). En effet, une étude a montré que l'inactivation de ce facteur dans le muscle squelettique de souris peut conduire à une diminution de 69 % de la densité capillaire (Wagner et al, 2006). Les niveaux d'ARNm et de protéines de VEGF semblent être réduits à l'état basal et suite à un exercice physique chez l'homme âgé par rapport à des individus jeunes (Gavin et al, 2007; Ryan et al, 2006). Ces

résultats sont plus ambigus chez la femme où seuls les niveaux protéiques de VEGF semblent être diminués à l'état basal (Croley et al, 2005). Etant donné que ce réseau de capillaires joue un rôle majeur dans l'apport en oxygène et en substrats, une altération de ce dernier peut alors perturber le fonctionnement musculaire.

Cependant, d'autres perturbations peuvent conduire à une diminution du flux sanguin, et notamment, le vieillissement semble associé à des perturbations de la vasoconstriction et de la vasodilatation des artérioles irriguant le muscle. En effet, certains travaux ont mis en évidence une augmentation de l'activité nerveuse sympathique, conduisant à une augmentation de la vasoconstriction musculaire de la jambe et aboutissant à une diminution du flux sanguin (Dinenno & Joyner, 2006). Cependant, ces effets ne sont pas retrouvés dans les muscles de l'avant-bras et semblent donc être dépendant du type de muscle (Dinenno et al, 2002 ; Nishiyama et al, 2008). En accord avec ces résultats, l'augmentation de l'endothéline-1, molécule induisant une vasoconstriction, a été observée chez l'homme âgé (Donato et al, 2009 ; Seals et al, 2011). En parallèle, la vasodilatation des vaisseaux sanguins semble, elle, réduite. Des études ont montré une diminution de la biodisponibilité de l'oxyde nitrique (NO), molécule produite par les cellules endothéliales vasculaires et induisant une vasodilatation des cellules musculaires lisses entourant les artères et artérioles (Muller-Delp et al, 2012; Muller-Delp et al, 2002 ; Taddei et al, 2000). De plus, l'augmentation des espèces oxygénées réactives (ROS, Reactive Oxygen Species) lors du vieillissement, ainsi que des altérations des voies de signalisation induisant une hyperpolarisation de l'endothélium vasculaire peut également limiter cette vasodilatation (Behringer & Segal, 2012).

Ainsi, ces changements du réseau vasculaire dans les muscles squelettiques peuvent contribuer à la diminution des capacités physiques qui apparait lors du vieillissement.

4. Vieillissement de la matrice extracellulaire

Les interactions entre la matrice extracellulaire (MEC) et les fibres musculaires déterminent les propriétés mécaniques du muscle squelettique (Purslow, 2002). Des changements de la structure, de la fonction et des propriétés biochimiques de la MEC contribuent à l'altération de la transmission de la force et de la réponse élastique passive du muscle squelettique observée avec l'âge avancé. Lors du vieillissement, une augmentation du tissu conjonctif est associée à la perte de la masse musculaire, ce qui contribue à la rigidité

musculaire et représente un handicap pour la contraction musculaire (Gao et al, 2008 ; Kragstrup et al, 2011).

Parmi les changements de la structure, il a été observé une augmentation du contenu en collagène, composant principal de la matrice extracellulaire, dans les muscles squelettiques âgés (Figure 28) (Hindle et al, 2009 ; Ramaswamy et al, 2011). Cependant, cette augmentation semble être dépendante du type de fibres musculaires où les fibres de type I semblent plus affectées par ces changements que les fibres rapides de type II (Zimmerman et al, 1993). Avec l'âge, on observe également une fixation plus importante de calcium par les hélices β des fibres élastiques présentes dans la MEC (Jacob, 2006). Ces fibres perdent alors leur élasticité, ce qui compromet la transmission de la force de tension aux tendons. De plus, une augmentation de l'infiltration lipidique dans le tissu conjonctif d'hommes âgés a été corrélée à une diminution de la force musculaire (Taaffe et al, 2009).

Les propriétés biochimiques de la MEC sont également affectées par le vieillissement et des changements de certains types de collagènes spécifiques ont été observés. En effet, le muscle âgé semble avoir une augmentation de la proportion relative de collagène I ainsi qu'une diminution du collagène de type III par rapport à un muscle jeune (Goldspink et al, 1994 ; Hindle et al, 2009). Ainsi, étant donné que les différentes isoformes de collagènes ont des caractéristiques fonctionnelles différentes, des altérations du réseau de collagène contribuent probablement à la détérioration des propriétés mécaniques du muscle vieillissant. De plus, le vieillissement est également associé à une augmentation des « cross-links » du collagène (Monnier et al, 2005; Rodrigues et al, 1996 ; Zimmerman et al, 1993), conduisant à une perturbation du maillage de la matrice, et en particulier de l'épimysium (Gao et al, 2008). On observe également la glycation non enzymatique progressive des groupements aminés (lysines en particulier) du collagène. Celle-ci est suivie par la formation de composés aromatiques cycliques désignés sous le nom de « advanced glycosylation end products » ou AGE (Bailey, 2001 ; Monnier et al, 2005). L'augmentation des « cross-links » du collagène et des AGEs lors du vieillissement contribue à l'augmentation de la rigidité de la MEC et altère la génération de la force lors de la contraction musculaire.

Enfin, lors du vieillissement musculaire, il apparait également des changements fonctionnels, et notamment il a été décrit une diminution de la transmission de la force latérale à l'environnement extracellulaire du muscle squelettique (Ramaswamy et al, 2011). Cette transmission de force latérale est permise par l'interaction entre la MEC et les

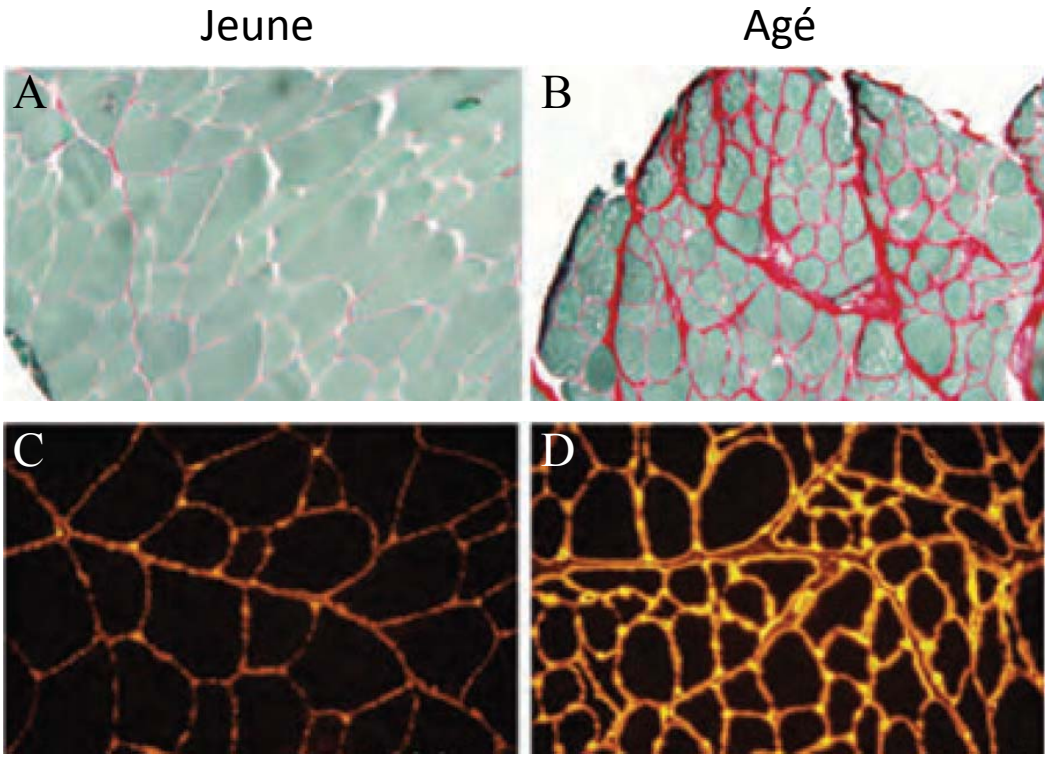

Figure 28: Coloration au rouge Sirius (A-B) et marquage du collagène IV (C-D) de coupes transversales de muscles de rats jeunes (A-C) et âgés (B-D). Ces marquages mettent en évidence l'épaississement du tissu conjonctif ainsi que l'augmentation du contenu en collagène associés au vieillissement.
D'après: **Ramaswamy** et al, 2011

costamères, et assure la protection des fibres musculaires contre les dommages liés à la contraction (Bloch & Gonzalez-Serratos, 2003). Ainsi, une diminution de cette force peut contribuer à l'augmentation des blessures et à une guérison plus lente chez les personnes âgées. Tout ceci suggère que les changements de la matrice extracellulaire des muscles squelettiques contribuent à la détérioration de la fonction musculaire lors du vieillissement, mais la contribution relative de ces différents changements est actuellement difficile à identifier.

III. Perturbations mitochondriales et accumulation lipidique intramusculaire lors du vieillissement

1. Mitochondries et vieillissement musculaire

Avec l'âge, il se produit une altération de la fonction mitochondriale ayant diverses conséquences, et notamment une diminution de la production énergétique, pouvant être associée à l'atrophie musculaire. En effet, des modifications biochimiques et bioénergétiques, associées à des changements de la dynamique mitochondriale, apparaissent lors du vieillissement (Peterson et al, 2012).

a- Changements biochimiques

Le vieillissement musculaire est associé à une augmentation de la production de ROS par la mitochondrie, incluant l'anion peroxyde $O_2^{\bullet-}$ et le peroxyde d'hydrogène H_2O_2 (Hutter et al, 2007). Ces derniers peuvent alors causer des dommages oxydatifs au niveau des structures environnantes, mais aussi au niveau de l'ADN mitochondriale, qui est à proximité du site de production des ROS. L'oxydation causée par les ROS conduit par la suite à l'apparition de protéines défectueuses, de lipides oxydés et de mutations de l'ADN mitochondriale (ADNmt), ce qui peut induire de nombreux dysfonctionnements cellulaires et mitochondriaux. Ces processus sont impliqués dans la théorie mitochondriale du vieillissement, qui soutient que l'accumulation des dommages causés par les ROS au fil du temps conduit à des altérations mitochondriales liées au vieillissement (Harman, 2006). L'accumulation de ces dommages oxydatifs peut être attribuée à une réduction de l'activité de

la chaîne de transfert des électrons, où ces derniers resteront plus longtemps au niveau des complexes I et III, augmentant ainsi la possibilité de donner des électrons à l'oxygène (Kushnareva et al, 2002). Cependant, il existe des enzymes dites « anti-oxydantes » capables d'éliminer ces ROS et de réduire l'état oxydé de la cellule, permettant de prévenir ces déficits liés à l'âge (Figure 29) (Lee et al, 2010 ; Johannsen & Ravussin, 2010).

En plus de l'augmentation de la production de ROS, le vieillissement est également associé à des altérations du contenu et de l'intégrité de l'ADNmt. Dans le muscle, plusieurs études ont montré une diminution du nombre de copies d'ADNmt (Lanza et al, 2008; Short et al, 2005 ; Welle et al, 2003), qui est accompagnée d'une augmentation de lésions de l'ADN. Une étude a montré que les délétions affectent plus de 70 % des molécules d'ADNmt dans le muscle squelettique de personnes âgées de plus de 80 ans (Chabi et al, 2005). L'impact fonctionnel de ces lésions est-il une conséquence ou la cause du vieillissement ? Cette question est encore largement débattue (Hiona & Leeuwenburgh, 2008). En effet, alors que certains ont montré qu'une diminution de la production énergétique mitochondriale se produit avant l'apparition de mutations de l'ADNmt (Conley et al, 2007), d'autres ont observé une corrélation forte entre le taux de mutations de l'ADNmt et l'atrophie des fibres musculaire ou une déficience bioénergétique (Bua et al, 2006; Wanagat et al, 2001). De plus, des travaux ont mis en évidence que des souris transgéniques, ayant un fort taux de mutations dans leur ADNmt, ont une quantité élevée de mitochondries anormales, un vieillissement prématuré, une sarcopénie sévère et une courte durée de vie (Hiona et al, 2010; Trifunovic et al, 2004).

Au niveau protéique, les résultats sont assez controversés. L'expression des protéines impliquées dans la glycolyse semble être inchangée ou diminuée avec l'âge, ce qui est en accord avec le shift du métabolisme glycolytique vers un métabolisme plus oxydatif observé chez la personne âgée (Gelfi et al, 2006). Cependant, les données obtenues sur l'expression des protéines impliquées dans le cycle de Krebs divergent et plusieurs auteurs ont montré une diminution de l'expression de ces protéines (Lanza et al, 2008 ; Picard et al, 2010; Piec et al, 2005 ; Short et al, 2005).

b- Changements bioénergétiques

Lorsque l'activité physique n'est pas prise en compte dans les critères de sélections des sujets, une diminution de l'activité enzymatique mitochondriale est observée lors du vieillissement. En effet, plusieurs études ont montré une diminution de l'activité des

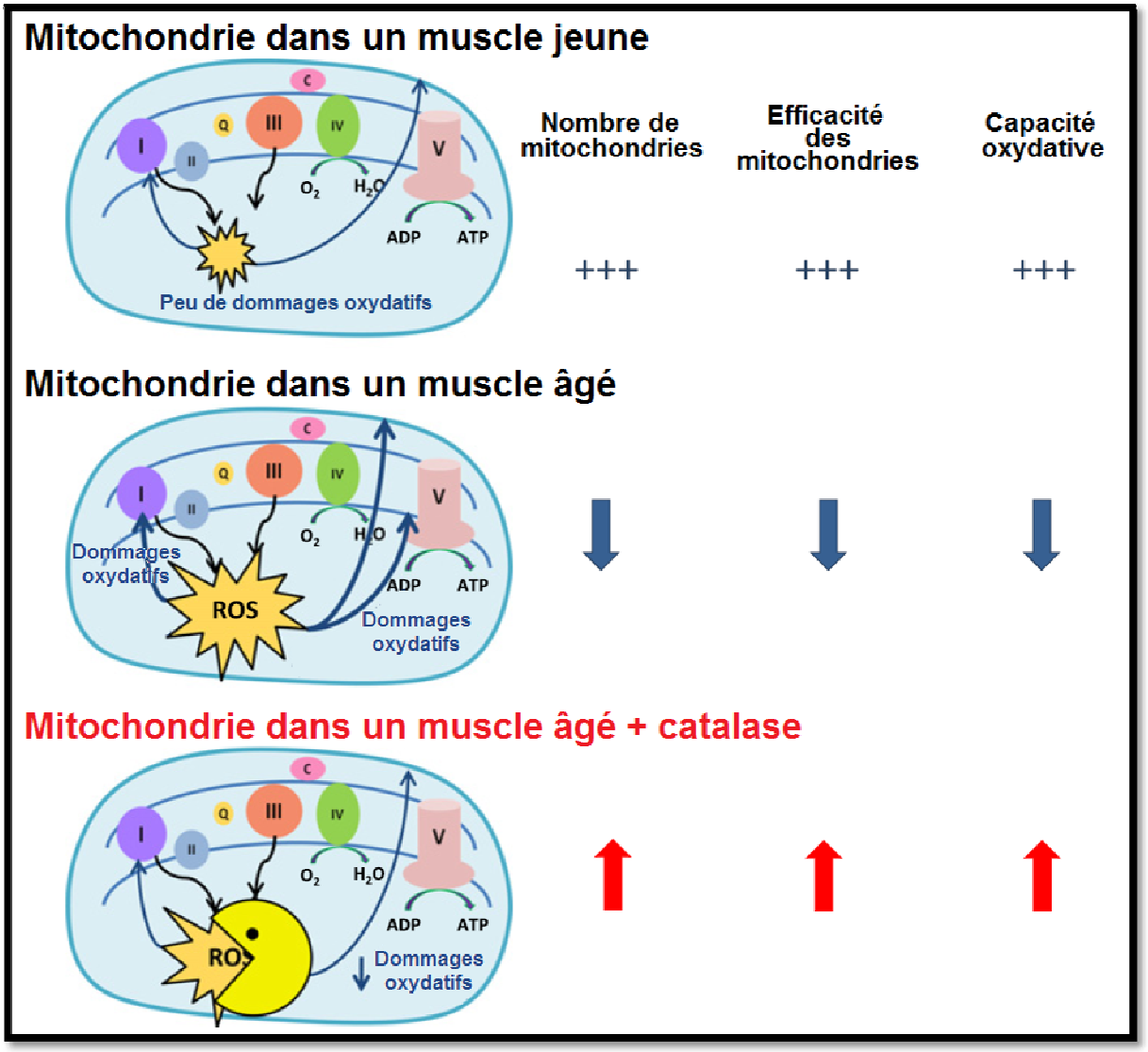

Figure 29: Altérations liées au vieillissement des mitochondries dans des muscles squelettiques de souris jeunes et âgées, et impact de la surexpression d'une enzyme anti-oxydante, la catalase.
D'après: **Johannsen** et al, 2010

complexes I et IV de la chaîne respiratoire (Figure 30) (Boffoli et al, 1994 ; Chabi et al, 2005 ; Crane et al, 2010). L'activité des enzymes impliquées dans le cycle de Krebs et la glycolyse est, elle, beaucoup plus discutée. Ceci peut être expliqué par l'impact différentiel du vieillissement en fonction du type du muscle étudié. En effet, les muscles lents ont une activité enzymatique mitochondriale plus affectée que les muscles rapides (Houmard et al, 1998). De plus, les différentes techniques d'isolation des mitochondries ou de normalisation des activités enzymatiques rendent difficile la comparaison entre ces études.

Ces changements enzymatiques peuvent, à leur tour, altérer la respiration mitochondriale ainsi que le flux d'ATP. En 2005, Short *et al*. ont mis en évidence une diminution d'environ 5 % tous les 10 ans de la capacité maximal de synthèse d'ATP par les mitochondries de muscles squelettiques (Short et al, 2005). De plus, chez la personne âgée, il a été observé une baisse de 50 % de la capacité oxydative par volume de muscle et une réduction de 30 % par volume de mitochondrie (Conley et al, 2000). Cependant, la plupart des altérations de la fonction mitochondriale liées au vieillissement semblent, en réalité, le résultat de l'inactivité physique. En effet, plusieurs études n'ont montré aucun changement de l'activité enzymatique mitochondriale, de la respiration ou du flux d'ATP lorsque les personnes âgées ont le même niveau d'activité physique que les personnes jeunes avec lesquelles elles ont été comparées (Lanza et al, 2008 ; Larsen et al, 2012; Safdar et al, 2010).

c- Changements de la dynamique mitochondriale

Une diminution de la biogenèse mitochondriale suggérée par la baisse de la densité de mitochondrie est observée lors du vieillissement du muscle squelettique (Conley et al, 2000 ; Crane et al, 2010). Un des principaux régulateurs de la biogenèse mitochondriale est PGC-1α (Peroxisome proliferator-activated receptor Gamma Coactivator alpha). Une surexpression de cette protéine dans des muscles squelettiques de souris augmente la capacité oxydative, supprime la dégradation mitochondriale et prévient l'atrophie musculaire (Wenz et al, 2009). Ainsi, il est tout à fait envisageable que la diminution des niveaux protéiques de PGC-1α, observée lors du vieillissement, soit une des causes pouvant expliquer la chute de la biogenèse mitochondriale (Conley et al, 2007 ; Zechner et al, 2010). La baisse de la densité de mitochondrie est également associée à des altérations des processus de fission et de fusion mitochondriales dans les muscles âgés (O'Connell & Ohlendieck, 2009). Ceci peut alors avoir pour conséquence une altération de l'intégrité de l'ADNmt, de la fonction respiratoire, de la

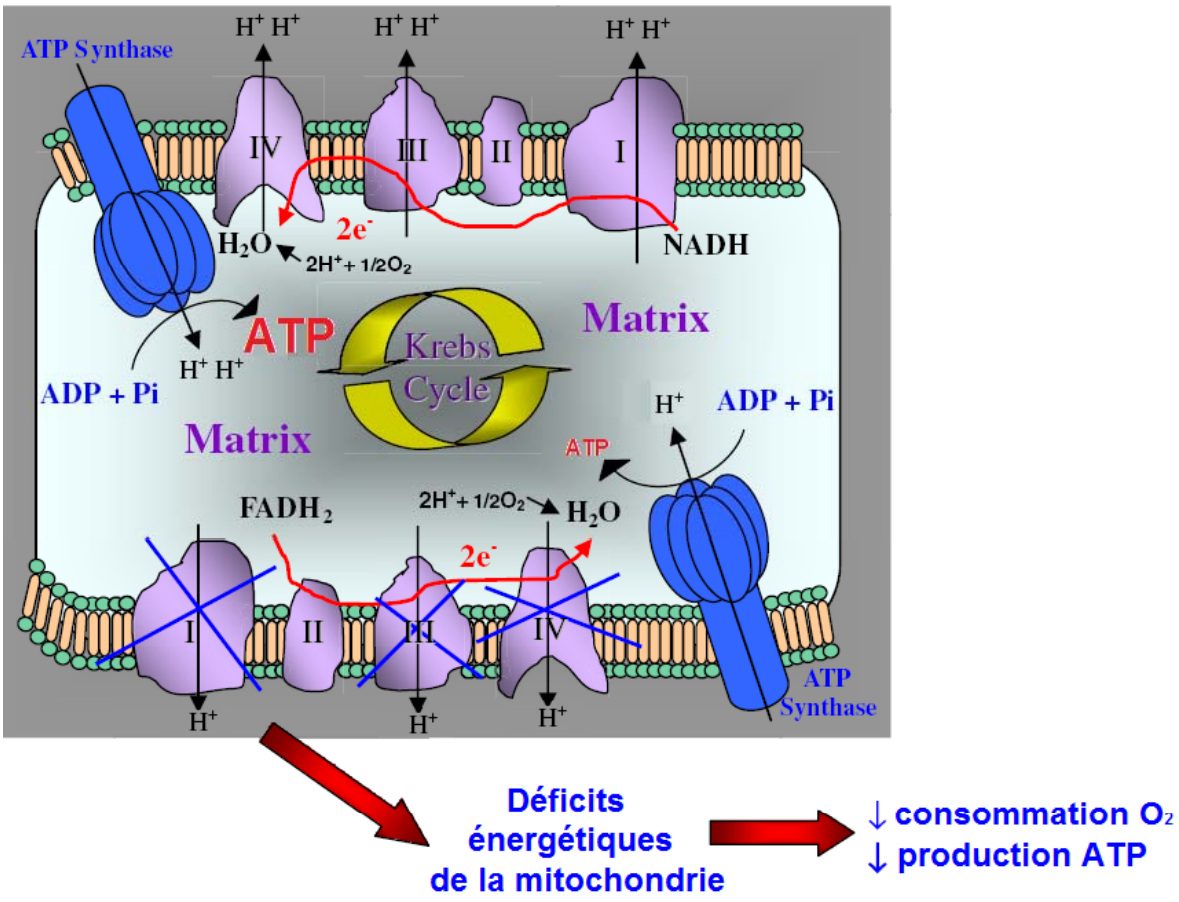

Figure 30: Conséquences de l'altération de l'activité des complexes I, III et IV de la chaîne respiratoire mitochondriale lors du vieillissement.
D'après: **Hiona & Leeuwenburgh**, 2008

production de ROS et de la sénescence cellulaire (Figure 31) (Lee et al, 2007 ; Marzetti et al, 2013).

Avec l'âge, le turnover mitochondrial est altéré (Calvani et al, 2013; Vina et al, 2009). Dans des conditions normales, la mitochondrie est dégradée par l'autophagie lysosomale, aussi appelée mitophagie (Wang & Klionsky, 2011). Lors du vieillissement, une diminution de l'autophagie a été observée dans des muscles squelettiques de rats âgés (Wohlgemuth et al, 2010). Les conséquences liées à cette baisse sont peu connues mais, comme la mitophagie est négativement corrélée à l'apoptose et aux dommages oxydatifs (Wohlgemuth et al, 2010), une réduction du flux autophagique peut contribuer à l'apparition de dysfonctions musculaires. De plus, un deuxième système va permettre de moduler le contrôle de la qualité et la dégradation mitochondriale, le système ubiquitine protéasome-dépendant ou UPS (en anglais, Ubiquitin-Proteasome System). Une altération de l'activité de ce dernier, observée lors du vieillissement, peut alors également contribuer à l'atrophie musculaire (Combaret et al, 2009 ; Strucksberg et al, 2010).

2. Accumulation de gouttelettes lipidiques intramyocellulaires et résistance à l'insuline

Plusieurs études ont mis en évidence une augmentation du contenu en lipides intramyocellulaires, stockés sous forme de gouttelettes lipidiques, dans le muscle squelettique lors du vieillissement (Crane et al, 2010; Cree et al, 2004 ; Petersen et al, 2003). Les mesures du contenu en triglycérides musculaires à partir de biopsies (Pan et al, 1997) ou par résonnance magnétique nucléaire (RMN) (Perseghin et al, 1999) ont montré une forte corrélation entre l'augmentation de la quantité de lipides intramyocellulaires et la résistance à l'insuline dans le muscle.

L'apparition de ces deux phénomènes lors du vieillissement peut être associée à l'atrophie musculaire. En effet, en plus de son rôle majeur dans l'homéostasie du glucose, l'insuline est également connue pour avoir une action anti-protéolytique et pour stimuler la synthèse protéique au niveau musculaire (Guillet & Boirie, 2005 ; Wilkes et al, 2009). Ainsi, l'insulino-résistance peut être considérée comme contribuant à la perte musculaire lors du vieillissement. En parallèle, les gouttelettes lipidiques, elles-mêmes, peuvent participer à la perte de masse du muscle squelettique. En effet, une étude a montré que l'expression d'une protéine associée aux LDs, la périlipine 2, était augmentée lors du vieillissement (Conte et al,

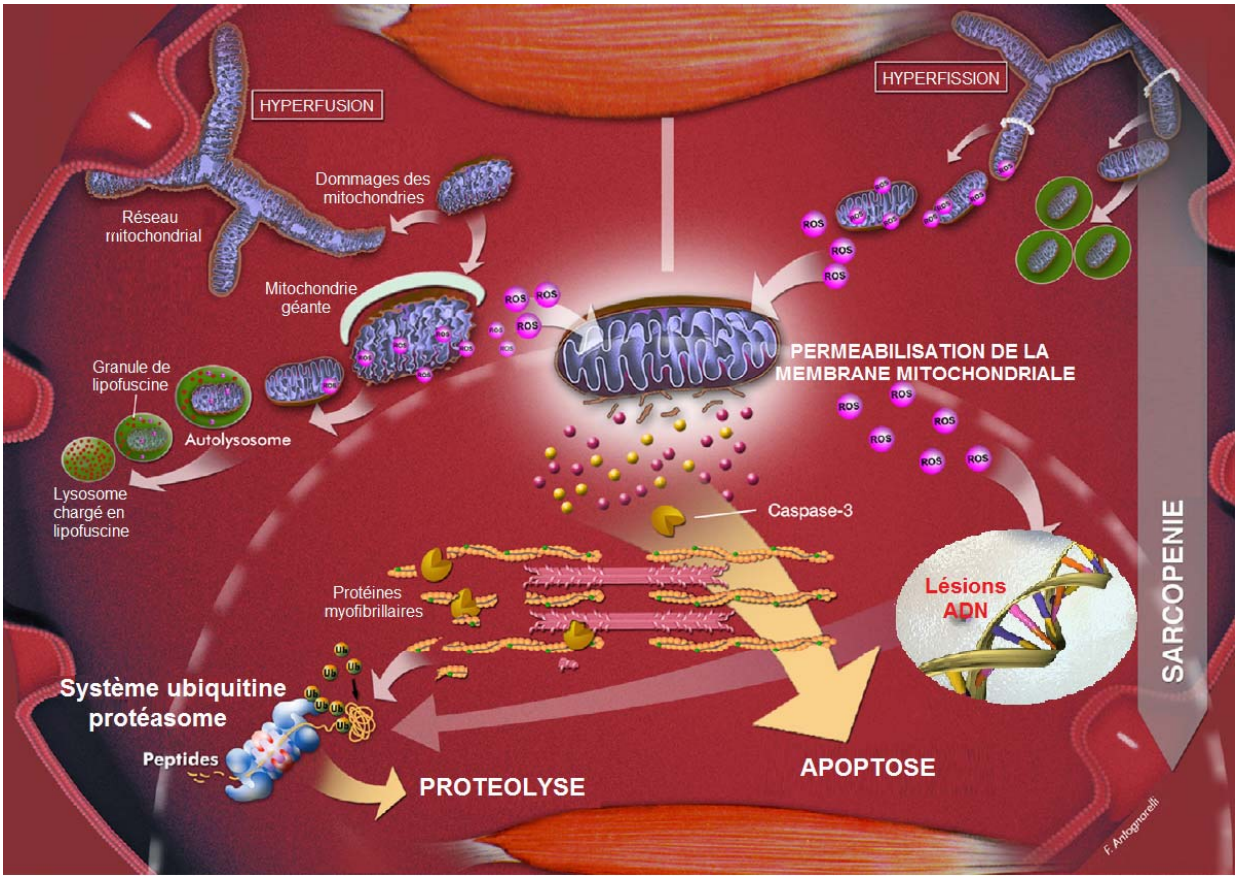

Figure 31: Scénario possible des altérations mitochondriales pouvant conduire à l'atrophie musculaire lors du vieillissement. Un déséquilibre de la dynamique mitochondriale conduit à une hyperfusion qui est associée à l'apparition de mitochondries géantes, caractérisées par une altération de la morphologie, une réduction de l'efficacité bioénergétique et une augmentation de la production de ROS. Une augmentation de lipofuscine dans les lysosomes contribue à des altérations de la voie lysosomale. Les ROS générés induisent également des altérations des structures environnantes, et notamment des lésions de l'ADN, pouvant perturber l'activité du système ubiquine-protéasome. De plus, une hyperfission peut conduire à une désintégration du réseau mitochondrial et à l'activation de la mitophagie. La génération de ROS par les fragments de mitochondries augmente potentiellement la fission, stimulant la dégradation des protéines musculaires et l'apoptose.
D'après: **Marzetti** et al, 2013

2013). Cette protéine est essentielle pour le stockage des lipides dans le muscle squelettique, mais son expression est inversement corrélée à la masse et à la force musculaire (Conte et al, 2013). Ainsi, la surexpression de cette protéine dans le muscle de personnes âgées peut contribuer à l'apparition de la sarcopénie.

De plus, l'augmentation de l'accumulation lipidique et l'apparition de la résistance à l'insuline semblent également associées aux perturbations du fonctionnement mitochondrial (Shulman, 2000). En effet, une étude a montré que, chez les personnes âgées, ces gouttelettes lipidiques ont une taille plus importante et sont moins fréquemment co-localisées avec les mitochondries dans le muscle squelettique (Crane et al, 2010). Ainsi, cette accumulation élevée de gouttelettes semble être associée à une réduction liée à l'âge de l'oxydation mitochondriale, conduisant à une augmentation de métabolites d'acides gras intracellulaires (diacylglycérol, fatty acyl Coenzyme A, céramides) qui vont perturber la signalisation de l'insuline (Lowell & Shulman, 2005; Petersen et al, 2003). En effet, une concentration élevée de ces métabolites peut induire l'activation d'enzymes à activité sérine/thréonine kinase, menant à des défauts dans la voie de signalisation de l'insuline dans le muscle, mais aussi dans le foie (Chow et al, 2010). Il en résulte, alors, une réduction de l'activité de transport de glucose stimulée par l'insuline, et une diminution de la synthèse de glycogène musculaire (Figure 32).

Ainsi, en plus de ses conséquences sur la perte musculaire, la résistance à l'insuline est aussi un des composants principal du syndrome métabolique, qui est un facteur de risque important pour l'apparition de maladies cardiovasculaires et du diabète de type II.

IV. Traitements et perspectives thérapeutiques

A ce jour, il n'existe aucun moyen de prévention efficace de la sarcopénie. Néanmoins, certaines actions permettent de retarder ou de freiner le déclin de la masse et de la fonction musculaire. Les causes potentielles de la sarcopénie sont nombreuses et certaines sont accessibles à des mesures thérapeutiques, notamment lorsque ces dernières sont mises en place précocement, avant que des altérations fonctionnelles trop importantes ne limitent les possibilités d'interventions (Guralnik, 2001).

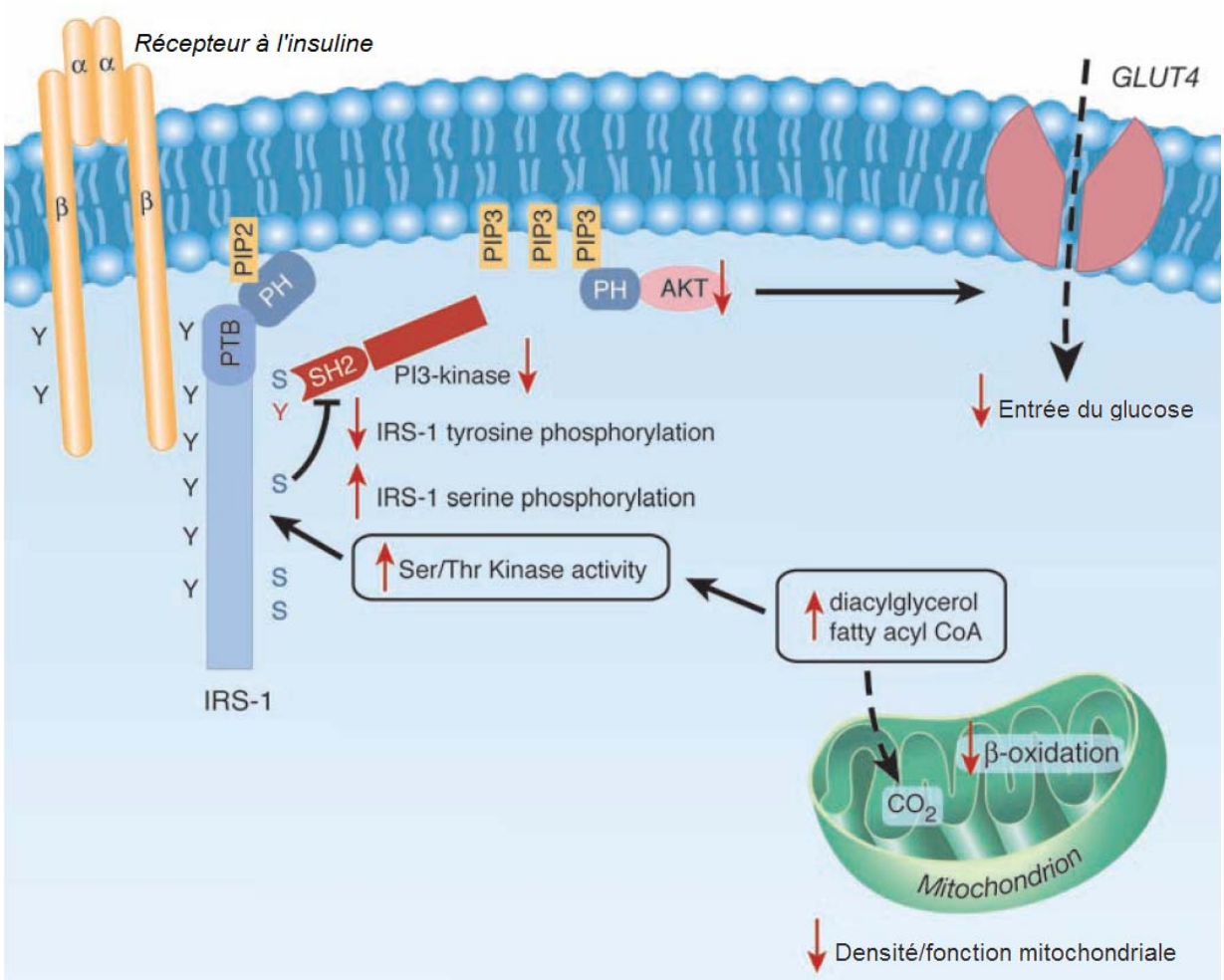

Figure 32: Mécanisme potentiel par lequel une altération de la fonction mitochondriale conduit à une résistance à l'insuline dans le muscle squelettique. Une diminution de l'oxydation mitochondriale des acides gras produit des niveaux élevés de fatty acyl CoA et de diacylglycérol. Ces molécules conduisent à l'activation d'enzymes à activité sérine kinase, menant à l'augmentation de la phosphorylation sérine d'IRS-1 (Insulin Receptor Substrate-1). Ceci aboutit à l'inhibition de l'activité de la phosphatidyl inositol 3-kinase (PI3-kinase). L'activité d'AKT (ou protéine kinase B) est alors diminuée et le transport du glucose stimulé par l'insuline est supprimé.

D'après: **Lowell & Shulman**, 2005

a- L'activité physique

Actuellement, aucune intervention ne s'est montrée aussi efficace que la pratique d'activités physiques dans la prévention et le traitement de la sarcopénie. L'activité physique entraîne une augmentation de la synthèse protéique (Churchward-Venne et al, 2013), mais également une diminution des signaux pro-apoptotiques dans le muscle squelettique, ce qui prévient l'atrophie des fibres musculaires (Song et al, 2006). Elle limite également les signaux pro-inflammatoires (Moon et al, 2012) et améliore la fonction mitochondriale (Peterson et al, 2012), ainsi que la vascularisation musculaire (Charifi et al, 2004 ; Park et al, 2012). L'exercice contribue enfin à prévenir certaines pathologies secondaires telles que l'athérosclérose, le diabète, l'ostéoporose et la prévalence des fractures (Karch et al, 2013; Layne & Nelson, 1999). Ainsi, l'exercice, même s'il ne permet pas de reprise de masse musculaire à court et moyen terme, améliore rapidement la qualité musculaire. Néanmoins, même chez les sujets âgés actifs, la fonte musculaire persiste. Ce phénomène est bien connu des sportifs de haut niveau qui constatent en vieillissant un déclin de leurs performances physiques malgré la poursuite d'un entraînement intensif.

b- Les traitements hormonaux

Le vieillissement du système endocrinien induit la chute de la production de nombreuses hormones dont les principales sont la testostérone, les œstrogènes, l'hormone de croissance GH, et le DHEA. Comme indiqué dans le Chapitre I de ce manuscrit, des traitements de supplémentation de ces hormones existent et ont, pour la plupart, montré des effets bénéfiques sur la masse et la force musculaire, ou encore sur la densité osseuse, la masse grasse et la sensibilité à l'insuline. Cependant, ces traitements montrent aussi beaucoup d'effets indésirables, comme l'apparition de cancers, et leur utilisation reste actuellement très controversée.

c- La nutrition

Chez les sujets âgés, une dénutrition protéino-énergétique est un frein au gain de masse et de force musculaire lors de programmes d'entraînement. Dans cette situation, une majoration des apports protéino-énergétiques ralentit la sarcopénie (Morais et al, 2006). A l'inverse, chez

des sujets ne présentant pas de dénutrition, les essais d'intervention ayant étudié l'effet d'une supplémentation protidique seule ou en association avec un programme d'entraînement physique se sont montrés, pour la plupart, inefficaces (Campbell & Leidy, 2007; Roth et al, 2000).

Les bienfaits de la restriction calorique sur le vieillissement, notamment sur le muscle squelettique, ont été démontrés (Lee & Min, 2013). A long terme, elle limiterait le stress oxydatif en diminuant la production de ROS par la mitochondrie, et par conséquent les dommages de l'ADN mitochondrial (Merry, 2002). Elle préviendrait également la perte de masse musculaire, la diminution du nombre de fibres musculaires et confèrerait une vasoprotection (McKiernan et al, 2004 ; Ungvari et al, 2010). Néanmoins, ces effets ne s'observent généralement qu'avec le maintien en parallèle de l'activité physique, et cette restriction reste difficile à appliquer dans notre mode de vie actuel. Afin de s'affranchir de ces difficultés, les gérontologues et biologistes se sont attachés à développer des drogues dont les effets miment ceux de la restriction calorique. On parle de CRM (en anglais, Caloric Restriction Mimetics). Trois drogues semblent avoir des effets similaires à la restriction calorique : le Resveratrol, la Rapamycine, la Metformine (Baur et al, 2006 ; Harrison et al, 2009 ; Onken & Driscoll, 2010). Cependant, des investigations plus approfondies doivent être réalisées afin de clarifier et de vérifier l'efficacité de ces potentiels CRM.

Chapitre IV : le syndrome métabolique

Le syndrome métabolique (SM) est en train de devenir un problème de santé publique majeur. Les facteurs de risques composant le syndrome métabolique sont assez bien décrits et incluent : une obésité abdominale, une dyslipidémie athérogène, une pression sanguine élevée, une résistance à l'insuline, ainsi qu'un état pro-inflammatoire et pro-thrombotique (Alberti et al, 2009; Grundy, 2007). L'ensemble ces facteurs est étroitement lié à la progression du diabète de type II, des maladies cardiovasculaires et d'autres maladies chroniques à l'échelle mondiale. Cependant, la définition de critères permettant le diagnostic du syndrome métabolique est actuellement débattue, et malgré les progrès réalisés dans notre compréhension de ce syndrome, sa pathophysiologie reste floue. Cela apparait clairement dans le développement, au cours des années, des différents concepts du syndrome, à travers les multiples tentatives visant à comprendre et démêler les interactions complexes entre les composants.

Etant donné que la prévalence du syndrome métabolique augmente fortement avec l'âge, le vieillissement semble ainsi jouer un rôle majeur dans l'apparition de ce syndrome. Cependant, malgré l'association étroite qui existe entre la résistance à l'insuline et l'atrophie musculaire liée à l'âge, les altérations du muscle squelettique chez les personnes âgées atteintes de syndrome métabolique sont peu connues.

Ce chapitre s'attache donc à décrire la prévalence et les différentes définitions données pour le diagnostic du syndrome métabolique, ainsi que les altérations du muscle squelettique connues et associées au syndrome métabolique chez l'adulte et lors du vieillissement.

I. Le syndrome métabolique : prévalence et conséquences

1. Prévalence du syndrome métabolique

La forte prévalence du syndrome métabolique est un phénomène mondial touchant entre 20 et 30 % de la population adulte (Grundy, 2008). Cependant, le calcul de la prévalence de ce syndrome dans les différentes régions du monde dépend de la définition des critères utilisés. Il est ainsi difficile de comparer les données de la littérature portant sur la prévalence

du syndrome métabolique. La figure 32 présente le résultat d'études sur la prévalence du syndrome métabolique dans divers pays (Cameron et al, 2004 ; Eckel et al, 2005). Ce graphique montre à quel point ces études diffèrent en fonction du design de l'étude, de la sélection des échantillons, de la définition du SM utilisée, de l'âge et du sexe de la population.

Cependant, la constatation la plus flagrante est que la prévalence du syndrome métabolique est fortement dépendante de l'âge. Ce phénomène est très clair en France où la prévalence est inférieure à 5 % chez les hommes et les femmes âgées entre 30 et 39 ans, puis augmente à 30 % chez les personnes âgées de 60 à 69 ans (Vernay et al, 2013). De façon similaire, dans une population iranienne, la prévalence passe de 10 % dans le groupe âgé de 20 à 29 ans, à 40 % dans le groupe de 60 à 64 ans (Azizi et al, 2003). Cet effet âge se retrouve également aux Etats-Unis où la prévalence augmente de 7 % chez les individus âgés de 20 à 29 ans, à 44 % et 42 % chez les individus âgés de 60 à 69 ans et de plus de 70 ans, respectivement (Ford et al, 2002) (Figure 33).

2. Troubles cardiovasculaires et diabète, les conséquences majeures du syndrome métabolique

L'importance du syndrome métabolique en termes cliniques et de santé publique reste incertaine. Un rapport sur sa valeur prédictive a conclu que ce syndrome pourrait être à l'origine d'environ 7 % des décès et de 17 % des maladies cardiovasculaires (Ford, 2005). Dans le même ordre d'idée, un rapport de la Framimgham Heart Offspring Study révélait que le syndrome métabolique contribuait au risque de maladie cardiovasculaire et de maladie coronarienne à raison de 34 % et de 29 % respectivement chez les hommes et de 16 % à 8 % respectivement chez les femmes (Wilson et al, 2005). Une méta-analyse récente de 37 études longitudinales a révélé une augmentation de 78 % du risque d'accident cardiovasculaire et de décès chez les personnes atteintes du syndrome métabolique (Gami et al, 2007).

En plus de ses conséquences sur l'apparition des troubles cardiovasculaires, le syndrome métabolique précède et prédit le diabète de type II. En 2004, une étude longitudinale a montré que les personnes atteintes de syndrome métabolique ont un risque de développer un diabète de type II qui est 5 fois supérieur à celui de personnes saines (Stern et al, 2004). De la même manière, une revue d'études prospectives a révélé que ce syndrome contribuait à plus de 52 % au risque de développer un diabète de type II (Ford, 2005).

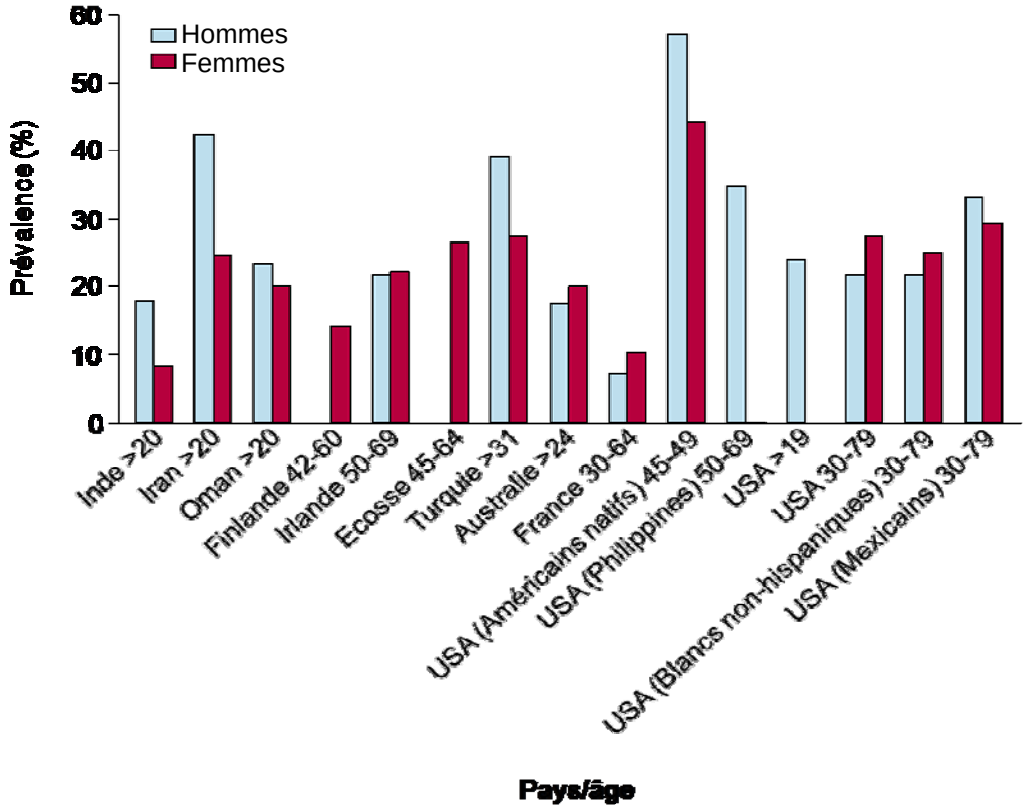

Figure 32: Prévalence du syndrome métabolique dans le monde.
D'après: **Cameron** et al, 2004

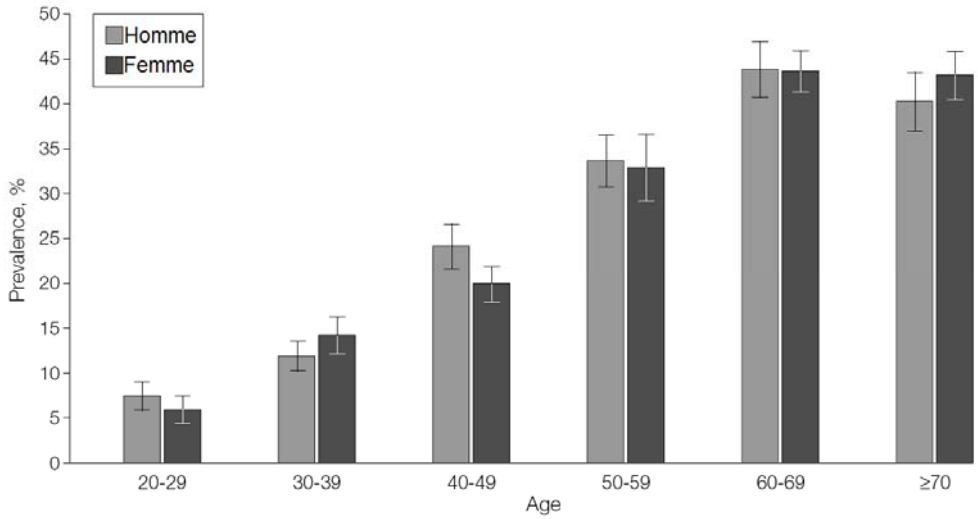

Figure 33: Prévalence du syndrome métabolique aux Etats-Unis en fonction de l'âge.
D'après: **Ford** et al, 2002

II. Evolution de la définition des critères de diagnostic du syndrome métabolique

1. Historique sur le syndrome métabolique

En 1988, Reaven a suggéré que l'insensibilité à l'insuline était responsable d'un ensemble de conditions, incluant une dyslipidémie, une hypertension et une hyperglycémie, qui constituaient un important facteur de risque de maladie cardiovasculaire. Cet ensemble de facteurs de risque sera appelé « syndrome X » (Reaven, 1988). Par la suite, d'autres auteurs l'ont baptisé « syndrome de résistance à l'insuline », « syndrome plurimétabolique », ou encore « syndrome dysmétabolique » (Ferrannini et al, 1991). Puis, en 1998, une définition du syndrome métabolique a été formulée par le groupe de travail sur le diabète de l'Organisation Mondiale de la Santé, aussi nommé WHO (World Health Organization) (Alberti & Zimmet, 1998). Modifiée un an plus tard par le groupe européen EGIR (European Group for Study of Insulin Resistance), cette définition s'accompagne d'une liste de critères destinés au diagnostic clinique (Balkau & Charles, 1999). Cependant, ces critères de diagnostics ont fait, et font toujours, l'objet de nombreux débats par les différents groupes de travail qui se sont succédés : le groupe NCEP ATP III (National Cholesterol Education Program Adult Treatment Panel III) en 2002, le groupe AACE (American Association of Clinical Endocrinologists) en 2003, la fédération internationale du diabète, ou IDF, et le groupe AHA/NHLBI (American Heart Association/National Heart, Lung, and Blood Institute) en 2005, ainsi que le groupe JIS (Joint Interim Statement) en 2009 (Alberti et al, 2009; Alberti et al, 2005 ; Einhorn et al, 2003 ; Grundy et al, 2005 ; NCPE/ATPIII, 2002).

2. Les définitions du syndrome métabolique : de 1998 à aujourd'hui

La définition du syndrome métabolique, donnée par le WHO en 1998, place la résistance à l'insuline comme un facteur de risque majeur et essentiel pour le diagnostic. Ainsi, un diagnostic de SM est alors basé sur la présence d'une insulino-résistance additionnée à deux autres facteurs de risques incluant, par exemple, une obésité centrale ou un taux de triglycérides trop élevé (Tableau 4). Les personnes atteintes de diabète de type II ne sont pas exclues de ce diagnostic. En 1999, le groupe EGIR modifie cette définition en se concentrant plus précisément sur l'obésité abdominale (Tableau 4). De plus, EGIR exclue les personnes

Groupe de travail	Définition en fonction des critères de diagnostics	Facteurs de risque et valeurs seuils
World Health Organization (WHO) (1)	Insulinorésistance + 2 autres facteurs de risque	• Insulinorésistance • Médicaments contre l'hypertension et/ou pression artérielle élevée (≥140/≥90 mmHg) • Triglycérides plasmatiques ≥150 mg/dL • HDL cholestérol <35 mg/dL chez l'homme et <39 mg/dL chez la femme • BMI >30 kg/m^2 et/ou ratio taille/hanche >0,9 chez l'homme et >0,85 chez la femme • Taux d'excrétion d'albumine urinaire ≥20 µg/min ou ratio albumine/créatine ≥30mg/g
European Group for Study of Insulin Resistance (EGIR) (2)	Résistance à l'insuline + 2 autres facteurs de risque	• Tour de taille Homme ≥94 cm Femme ≥80 cm • Hypertention ≥140/≥90 mmHg • Triglycérides plasmatiques ≥150 mg/dL ou HDL cholestérol <39 mg/dL chez l'homme et la femme • Glucose plasmatique élevé sans présence de diabète
National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATPIII) (3)	Présence de 3 facteurs de risque sur 5	• Tour de taille Homme >102 cm Femme >88 cm • Triglycérides ≥150 mg/dL • HDL cholestérol <40 mg/dL Homme <50 mg/dL Femme • Pression artérielle ≥130/≥85 mmHg • Glycémie à jeun ≥110 mg/dL
American Association of Clinical Endocrinologists (AACE) (4)	Nombre de facteurs de risque non requis, jugement clinique	• Surpoids/Obésité BMI ≥25 kg/m^2 • Triglycérides ≥150 mg/dL • HDL cholestérol <40 mg/dL Homme <50 mg/dL Femme • Pression artérielle ≥130/≥85 mmHg • Taux de glucose 2 heure après injection >140 mg/dL • Glycémie à jeun entre 110 et 126 mg/dL • Autres facteurs de risques : *Antécédents familiales de diabètes de type II, hypertension ou maladies cardiovasculaires* *Syndrome ovarien polykystique* *Sédentarité* *Age*

Tableau 4: Définitions des différents critères de diagnostic du syndrome métabolique décrits par les divers groupes de travail.
D'après: Alberti et al, 1998 (1), Balkau et al, 1999 (2), NCEP/ATPIII report, 2002 (3) et Einhorn et al, 2003 (4).

diabétiques car la résistance à l'insuline est vue comme un facteur de risque primaire. Cette définition a été suivie par une définition plus simple donnée par le NCEP ATPIII, qui diffère sur plusieurs points par rapport à celle donnée par le WHO. Le NCEP ATPIII propose ainsi qu'un diagnostic de syndrome métabolique peut être fait lorsqu'au moins 3 des 5 critères présentés dans le tableau 3, sont présents. Le NCEP ATPIII n'estime pas la résistance à l'insuline comme un facteur de risque indispensable pour le diagnostic du syndrome métabolique. Par contre, ce groupe place l'obésité centrale comme étant un facteur de risque majeur et la quantifie *via* la mesure du tour de taille. De plus, cette définition considère que le taux d'HDL et de triglycérides sont deux composants du SM distincts puisqu'ils peuvent conduire à une dyslipidémie athérogène de façon indépendante. Par la suite, en 2003, l'AACE préfère utiliser le terme de « syndrome de résistance à l'insuline » plutôt que SM. Ce groupe ne spécifie aucun nombre particulier de critères, le diagnostic se faisant suite à un jugement clinique, et les patients atteints de diabètes de type II sont exclus (Tableau 4).

Parmi toutes ces définitions proposées, la communauté scientifique préfère l'utilisation de celle donnée par le NCEP ATPIII, puisqu'elle présente l'avantage d'être simple et facilement applicable cliniquement. Cependant, un débat s'est ouvert sur les valeurs de seuil du tour de taille. En effet, plusieurs chercheurs affirment que ce seuil ne peut pas être appliqué à tous les pays. C'est ainsi, qu'en 2005, l'IDF détermine différentes valeurs limites de tour de taille en fonction de l'ethnie et du sexe. De plus, l'IDF définit le tour de taille comme étant un critère indispensable au diagnostic du syndrome métabolique. Cette affirmation est contredite, la même année, par le groupe AHA/NHLBI. Il faut alors attendre 2009 pour qu'une déclaration intermédiaire conjointe (JIS) entre ces différents groupes de travail propose de définir le syndrome métabolique *via* la définition donnée par le NCEP ATPIII, mais avec une valeur seuil du tour de taille qui diffère en fonction des pays et du sexe (Tableau 5). C'est actuellement cette définition qui est communément admise dans la littérature (Tableau 6).

III. Stratégies thérapeutiques

Le principal objectif chez les personnes atteintes du syndrome métabolique est de réduire le risque de développer le diabète, une maladie cardiovasculaire ou d'autres maladies chroniques. Dans un premier temps, l'accent doit être mis sur la modification des facteurs de risque sous-jacents (insensibilité à l'insuline, obésité, inflammation) à travers une adaptation du mode de vie (intensification de l'activité physique, alimentation cardioprotectrice et perte

Population	Valeurs seuil de tour de taille pour l'obésité abdominale	
	Homme	**Femme**
Europoïde	≥94 cm	≥80 cm
Caucasienne	≥94 cm (risque élevé) ≥102 cm (risque très élevé)	≥80 cm (risque élevé) ≥88 cm (risque très élevé)
Etats-Unis	≥102 cm	≥88 cm
Canadienne	≥102 cm	≥88 cm
Européenne	≥102 cm	≥88 cm
Asiatique (dont Japon)	≥90 cm	≥80 cm
Asiatique	≥90 cm	≥80 cm
Japonaise	≥85 cm	≥90 cm
Chinoise	≥85 cm	≥80 cm
Méditerranéenne	≥94 cm	≥80 cm
Africaine Sub-saharienne	≥94 cm	≥80 cm
Amérique du Sud	≥90 cm	≥80 cm

Tableau 5: Recommandations actuelles des valeurs seuil de tour de taille pour déterminer l'obésité abdominale en fonction du sexe et de la population.
D'après: Alberti et al, 2009.

Facteurs de risque	Valeurs seuils
Tour de taille élevé	Cf valeurs définies dans le Tableau 4
Triglycérides (ou traitement contre l'hypertriglycéridémie)	≥150 mg/dL
HDL cholestérol	<40 mg/dL chez l'homme <50 mg/dL chez la femme
Pression artérielle (ou traitement contre l'hypertension)	≥130/≥85 mmHg
Glycémie à jeun (ou traitement contre l'hyperglycémie)	≥100 mg/dL

Tableau 6: Définition des critères de diagnostics du syndrome métabolique admis actuellement.
D'après Alberti et al, 2009.

de poids). Un régime alimentaire a d'ailleurs été proposé afin de limiter l'apparition de l'athérogenèse, et doit contenir moins de 7 % d'acides gras saturés, moins de 1 % d'acides gras trans et moins de 200 mg/jour de cholestérol. Ce régime pourrait également réduire la pression artérielle (Grundy, 2007). Un programme d'entraînement physique, consistant en une marche de 40 à 50 minutes trois fois par semaine durant 12 semaines, a également fait ses preuves (Colombo et al, 2013). En effet, cet entraînement a permis de réduire le tour de taille et d'améliorer les capacités cardiovasculaires.

Un traitement pharmacologique spécifique peut s'avérer adéquat pour traiter les composants cliniquement significatifs du syndrome tels que l'hypertension, la dyslipidémie ou l'hyperglycémie. Cependant, l'intérêt d'adapter un traitement à une combinaison spécifique de critères n'a pas été étudié.

IV. Syndrome métabolique et effet sur le muscle squelettique âgé

L'augmentation de la prévalence du syndrome métabolique chez les personnes âgées peut être expliquée par l'altération des différentes fonctions physiologiques observées lors du vieillissement, et plus particulièrement par les altérations du muscle squelettique. En effet, les altérations du muscle âgé, et notamment l'apparition de l'insulino-résistance, semblent être fortement associées au syndrome métabolique. Ceci a été confirmé par une étude de Petersen *et al*., en 2007, qui a mis en évidence que l'insulino-résistance dans le muscle squelettique induit une dyslipidémie athérogène, ce qui favorise le développement du SM (Petersen et al, 2007).

De plus, plusieurs études ont récemment mis en évidence qu'une perte de masse et/ou de force musculaire est fréquemment observée chez les individus âgés atteints de SM (Jurca et al, 2005 ; Moon, 2013; Yang et al, 2012). En plus de la perte de force, une étude a également montré que le syndrome métabolique était associé à une diminution des capacités fonctionnelles musculaires (Vieira et al, 2013). Cependant, malgré la corrélation qui semble exister entre vieillissement, muscle et SM, les altérations du muscle squelettique chez les personnes âgées atteintes de syndrome métabolique sont peu connues.

Conclusion et objectifs de la thèse

Le vieillissement est caractérisé par la détérioration de nombreuses fonctions biologiques : adiposité élevée, fragilité osseuse et articulaire, altérations des systèmes nerveux, cardiovasculaires, endocriniens et immunitaires. Le vieillissement musculaire, aussi appelé sarcopénie, constitue en fait un élément central pour la régulation de nombreuses fonctions physiologiques, puisqu'il conduit à une perte d'autonomie fonctionnelle et à une moindre capacité à lutter contre les pathologies et les agressions métaboliques. En effet, le tissu musculaire est un organe important impliqué dans la contraction, mais également dans le stockage des protéines, dans l'homéostasie du glucose et des lipides, ainsi que dans la communication cellulaire.

Manifestement, le vieillissement musculaire est un phénomène complexe et multifactoriel, dont les mécanismes restent mal connus. Il est caractérisé par des modifications de la structure et de la composition des fibres musculaires, par une diminution de son potentiel régénératif, une altération de la vascularisation et un épaississement de la matrice extracellulaire. De plus les changements du muscle squelettique liés à l'âge conduisent également à des dysfonctionnements de la fonction mitochondriale associés à une augmentation de l'accumulation des lipides intramusculaires, ce qui contribue au développement de la résistance à l'insuline chez la personne âgée. Cependant, les mécanismes conduisant à la sarcopénie restent encore mal connus, et de plus amples investigations sont nécessaires afin d'identifier de nouvelles voies permettant, à terme, de développer des outils préventifs et thérapeutiques pour « mieux vieillir ».

En plus de la perte de masse et de force musculaire, le vieillissement est associé à l'apparition de nombreuses pathologies, dont le syndrome métabolique est probablement l'une des plus prévalentes. Ce syndrome pourrait être à l'origine de 7 % des décès, de 17 % des maladies cardiovasculaires, et augmente de 5 fois le risque de développer un diabète de type II. Plusieurs études ont récemment mis en évidence qu'une perte de masse et/ou de force musculaire est fréquemment observée chez les individus âgés atteints de syndrome

métabolique. Cependant, les altérations physiologiques et moléculaires du muscle squelettique chez les personnes âgées atteintes de SM sont peu décrites dans la littérature.

Ainsi les objectifs de la thèse ont été :

1- **Identifier de nouveaux biomarqueurs du vieillissement musculaire chez la femme post-ménopausée** *via* l'utilisation de techniques à haut débit telle que la protéomique. Le différentiel d'expression de plusieurs des protéines identifiées a ensuite été confirmé par la technique de Western-Blot (**Publication n°1**, *en cours de soumission* ; **Publication n°2**, *publiée dans Mol Cell Proteomics*).

2- **De mettre en évidence les altérations du muscle squelettique chez l'homme âgé sain ou atteint de syndrome métabolique**. La composition, la structure, l'activité enzymatique oxydative et le contenu lipidique des différents types de fibres musculaires ont été quantifiés (**Publication n°3**, *en cours de soumission*). Par la suite, les modifications du réseau vasculaire et de la matrice extracellulaire dans le muscle squelettique ont également été observées (**Publication n°4**, *en préparation*). Une étude protéomique a également été réalisée afin de **rechercher de nouveaux biomarqueurs du vieillissement musculaire associé ou non au syndrome métabolique** (**Publication n°5**, *en préparation*).

Résultats expérimentaux

I. Etude protéomique différentielle du vieillissement musculaire chez la femme post ménopausée

1. Introduction

Le vieillissement musculaire conduit à une perte progressive de masse et de force musculaire, aussi appelée sarcopénie (Bijlsma et al, 2013a), contribuant à une moindre autonomie des personnes âgées mais également à l'augmentation du risque de chute et à une diminution de la résistance contre les agressions métaboliques (Szulc et al, 2010).

Les changements liés à l'âge sont caractérisés par des altérations de la morphologie, de la fonction et des propriétés biochimiques du muscle squelettique. Bien que toutes ces altérations aient été bien décrites dans la littérature, les mécanismes moléculaires restent encore mal connus. Le profil d'expression des ARNm constitue un premier niveau d'information essentiel mais le profil d'expression des protéines est indispensable. En effet, contrairement au génome, le protéome varie en réponse à de nombreux facteurs physiologique ou pathologiques. L'analyse des changements liés au vieillissement du protéome musculaire peut être réalisée *via* l'utilisation de différentes approches complémentaires: l'électrophorèse bidimensionnelle (2DGE) et le « Shotgun ». La technique de 2DGE permet de mettre en évidence des variations au niveau des différentes isoformes d'une protéine. Cependant, étant donné que la 2DGE consiste à séparer les protéines en fonction de leur point isoélectrique (pI) et de leur poids moléculaire (PM), cette approche ne permet d'analyser qu'une portion réduite du protéome et est défaillante pour les PM et les pI extrêmes ainsi que pour les protéines membranaires. A l'inverse, le Shotgun implique l'hydrolyse des protéines et l'analyse des peptides issus de cette hydrolyse. Le Shotgun évite ainsi les limites liées aux PM et pI extrêmes et permet d'avoir une vision plus globale. Néanmoins, cette technique possède également certains inconvénients comme la perte de la notion d'isoformes ainsi qu'une augmentation importante de la complexité.

Bien que plusieurs analyses protéomiques par 2DGE aient été réalisées sur le muscle squelettique de rats âgés (Capitanio et al, 2009 ; Doran et al, 2008 ; Gannon et al, 2009 ; O'Connell & Ohlendieck, 2009; Piec et al, 2005), peu d'études ont été menées chez l'homme et les résultats sont assez contradictoires (Gelfi et al, 2006 ; Short et al, 2005 ; Staunton et al, 2012). De plus, à notre connaissance, aucune analyse par Shotgun n'a été réalisée sur le muscle squelettique lors du vieillissement chez l'homme ou chez la femme. Le but de ce travail a donc été d'identifier de nouveaux biomarqueurs potentiels du vieillissement musculaire chez la femme âgée post-ménopausée, *via* une analyse protéomique par 2DGE et par Shotgun.

2. Résultats principaux

➢ Etude protéomique du vieillissement musculaire par électrophorèse bidimensionnelle (**Publication n°1**)

Au cours de cette étude, différentes gammes de pH ont été utilisées afin d'optimiser la séparation des protéines. De plus, afin d'améliorer la détection des protéines les moins abondantes, nous avons effectué une extraction des protéines musculaires à faible force ionique, ce qui permet d'éliminer les protéines myofibrillaires de l'échantillon. Parmi un total de 1919 spots communs entre tous les individus, 106 sont exprimés différentiellement et ont été identifiés par spectrométrie de masse (nanoLC-MS/MS), ce qui correspond à 73 protéines différentes. Les résultats indiquent d'importantes modifications du métabolisme énergétique (ex, glycérol-3-phosphate déshydrogénase, créatine kinase, pyruvate déshydrogénase, énolase), qui peuvent être liées à une diminution de la production d'ATP par le muscle squelettique et donc à une capacité réduite de générer la force musculaire chez les personnes âgées. Une fraction de protéines différentielles sont associées aux protéines de myofibres (chaînes légères de myosines, troponine T, vinculine), ce qui pourrait en partie expliquer l'altération des propriétés contractile lors du vieillissement. De plus, nous avons également observé des modifications au niveau de la transduction du signal liée au calcium (sarcaluménine, calséquestrine, annexines) et au niveau de la protéolyse (ex, VCP). Pour conclure, plusieurs de ces biomarqueurs n'avaient pas été identifiées comme étant

différentiellement exprimés lors du vieillissement du muscle squelettique et représentent de nouvelles pistes afin de mieux comprendre les mécanismes moléculaires mis en jeu.

- Etude protéomique du vieillissement musculaire par shotgun (**Publication n°2**)

Lors d'une précédente analyse par shotgun réalisée à partir d'extraits de protéines musculaires totales, les isoformes majeures des chaînes lourdes de myosine constituaient environ 42% du spectre total (Hojlund et al, 2008). Etant donné que ces isoforms peuvent perturber l'identification des autres protéines, les protéines myofibrillaires ont été précipitées à faible force ionique et nous nous sommes intéressés à la fraction soluble. Les résultats de cette étude protéomique ont montré que, parmi 366 protéines identifiées et communes à tous les individus, 35 semblent associées au vieillissement musculaire. La majorité de ces protéines interviennent au niveau du métabolisme énergétique (furamate hydratase, aspartate aminotransférase, ATP synthase) ou au niveau des myofilaments et du cytosquelette (ANKRD2, cofilin-1), et constitue de nouveaux biomarqueurs potentiels de la sarcopénie.

Il est intéressant de noter que les approches 2DGE et shotgun ne possèdent que 6 protéines différentielles communes. Du fait de méthodologies différentes, l'une étant basée sur la séparation des protéines et l'autre sur la séparation des peptides issus de l'hydrolyse des protéines, ces deux techniques s'avèrent complémentaires dans l'analyse du protéome. Globalement, ces travaux constituent la plus large analyse des variations du protéome musculaire liées à l'âge et permettent d'identifier 98 biomarqueurs potentiel du vieillissement du muscle squelettique.

Publication 1

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“Proteomics of muscle aging in women”

En cours de soumission

Proteomics of muscle aging in women

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KEYWORDS : Skeletal muscle, aging, post-menopausal women, proteomics.

ABSTRACT : Muscle aging (sarcopenia) contributes to both loss of autonomy and increased morbidity, but the mechanisms involved are complex and likely result from the alteration of a variety of interrelated functions. In order to better understand the molecular mechanisms underlying muscle aging, we have undertaken a top-down differential proteomic approach to identify novel biomarkers. Muscle samples were compared between adult (54 ± 4 years) and old (78 ± 2 years) post-menopausal women. Total and low-ionic strength extracts were prepared for each muscle and each individual was assessed separately. Two-dimensional gel electrophoreses (2DGE) with overlapping IPGs were used to improve the separation of muscle proteins. Overall 1919 protein spots were matched between all individuals, 106 were differentially expressed and identified by mass spectrometry (nanoLC-MS/MS), and they corresponded to 73 different proteins. Results indicated important modifications in energy metabolism (i.e. glycerol-3-P dehydrogenase, creatine kinase, pyruvate dehydrogenase, β-enolase, cytochrome b-c1 complex subunit Rieske), which may be related to dysfunctions in old muscle force generation. A fraction of the differentially expressed proteins were linked to myofiber proteins (myosin light chains, troponin T, vinculin), which may account for alterations in contractile properties. As previously described, we observed differential regulation of several proteins implicated in cytoprotection and detoxification like heat shock proteins or aldehyde dehydrogenase. We further noticed modifications of calcium-related signal transduction (sarcalumenin, annexins) and proteolysis (VCP). To conclude, several biomarkers of these proteomic analyses were previously unrecognized as differentially expressed in old muscles, and may represent novel starting points to elucidate some mechanisms of sarcopenia.

Introduction

Aging affects most tissues and physiologic functions, and one of the most affected organs by aging is the skeletal muscle. The progressive decline in muscle mass and function due to aging, which is also referred to as sarcopenia [1], contributes to both loss of autonomy, increased prevalence for falls and decreased resistance to metabolic aggression that increase morbidity [2]. Numerous theories have been proposed to explain sarcopenia. Obviously, this is a multifactorial phenomenon which implicates intrinsic factors such as perturbations in the endocrine system, neuronal remodelling, oxidative stress and deficiencies in muscle regeneration, extrinsic factors such as diet and exercise, and also probably other unknown mechanisms [3].

Age-related degenerative changes are reflected in alterations in muscle morphology, function, and biochemical properties. Muscle aging is thus associated with muscle fiber atrophy [4], reduced muscle regenerative capacity [5], and neuropathic processes leading to motor unit denervation [6]. Mitochondrial dysfunctions with decreased capacity of oxidative enzymes and a decline of mitochondrial ATP production are also observed with aging in skeletal muscles [7, 8].

The overall functional, structural, and biochemical alterations in aging muscle have been extensively studied, but the molecular mechanisms implicated remain to be specified. The differential expression profiles of mRNAs constitute a first essential level of information but analysis of the expression profile of proteins in aging is also required to understand the molecular mechanisms important for the muscle aging process [9]. In fact, unlike the genome, the proteome varies in response to many physiological or pathological factors. In addition, the proteome is orders of magnitude more complex than the transcriptome due to post-translational modifications, protein oxidation or limited protein degradation [10].

Several studies have been conducted in rat muscle and proteomic profiling has demonstrated substantial alterations in muscle proteins involved in key metabolic pathways, myofibrillar remodelling, cytoskeleton organisation and mechanisms of cytoprotection and cytodetoxification [11-15]. However, few studies have been conducted with human muscle and results are contradictory. Gelfi et al. [16] have shown that several enzymes involved in oxidative metabolism, including ubiquinol-cytochrome c reductase or aspartate aminotransferase, were more abundant in elderly than in young people, while Short et al. [8] have demonstrated a decrease in these enzymes with aging. In contrast, some results observed in rat muscle proteomic analyses were confirmed in human muscle, such as a decrease in enzymes involved in glycolytic metabolism and an increase in proteins involved in cytoprotection (carbonic anhydrase 3) and cytodetoxification (Hsp70) [16, 17].

Because epidemiological studies have indicated accelerated muscle wasting after the fifth decade with an approximately 2% reduction in muscle mass per year [18], we have undertaken a top-down differential proteomic approach by two dimensional gel electrophoresis (2DGE) to determine potential changes after the fifth decade of life and to identify novel biomarkers of muscle aging. We have performed 2DGE from biopsies of *vastus lateralis* from middle aged (56 years) *vs.* aged (78 years) women and for better separation of proteins, two different strategies

were conducted. First, overlapping Immobilized pH Gradient (IPGs) with three different pH ranges were used to improve muscle proteins separation, and secondly we assessed low salt extracts to discard myofibrillar proteins and improve the detection of low abundance proteins. The proteomic profiling of aged skeletal muscle fibers revealed a differential expression pattern for metabolic enzymes, contractile element and cytoskeletal proteins, calcium signaling, stress proteins, regulation of protein misfolding, but also for components of proteolytic systems, such as Valosin-containing protein (VCP) and calpain small unit 1 (CAPNS1) with aging. The present study demonstrates that a severe perturbed protein expression pattern is associated with alterations of muscle function in elderly and we have identified new potential biomarkers of sarcopenia that had not been previously described.

Materials and Methods

Subjects. Patients were included in the Leiden University Medical Center (Leiden, The Netherlands) and Rijnland Hospital (Leiderdorp, The Netherlands) between June 2010 to September 2012. Exclusion criteria consisted of previous knee or hip surgery (with the exception of arthroscopy), rheumatoid disease, diabetes mellitus, use of oral corticosteroids, and metastasized malignant. Twenty four post-menopausal women undergoing hip surgery for hip arthrosis were selected in the present study and divided in two groups: adult women aged 56.4 ± 1.3 years (n = 11) and old women aged 78.3 ± 0.5 years (n = 13). The study was approved by the medical ethical committees (n° P10.060) of Leiden University Medical Center and the Rijnland Hospital, and was performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all patients. The adult subjects were more active than elderly, but not involved in any specific training program. The activity score, based on self-report in a questionnaire, was significantly higher for adult than for old women ($p < 0.035$). Adult women were cycling more than old women (83% and 36 %, respectively), and adult women had less walking aid than old women (33% vs. 71%). The adult and old groups presented similar body mass index (24.5 $\pm$ 1.4 kg/m^2 and 26.2 $\pm$ 1.2 kg/m^2, respectively). Muscle samples were obtained by surgical biopsy from the *vastus lateralis* muscle and were immediately frozen in liquid nitrogen and stored at -80°C until used.

Reagents. Acrylamide, bisacrylamide and Hybond^{TM}-P membrane were purchased from Amersham Bioscience/GE Healthcare (Little Chalfont, UK). Immobilized pH gradient (IPG) buffers, 18 cm ReadyStrip IPG strips (pH 5-8),18 cm ReadyStrip IPG strips (pH 3.0-5.6), 18 cm ReadyStrip IPG strips (pH 5.3-6.5), and Electrode Wicks were from Bio-Rad Laboratories (Marnes la Coquette, France), and orthophosphoric acid, ammonium sulphate and absolute ethanol were from VWR (Strasbourg, France). All other chemicals were from Sigma (L'Isle-d'Abeau Chesnes, France). Sequence grade-modified trypsin was purchased from Promega (Charbonnières-les-bains, France), and Luminata^{TM} Western Horseradish peroxidase (HRP)

substrate and ReBlot Plus Strong antibody stripping solution were from Millipore (Molsheim, France). For immunoblotting, the monoclonal antibodies against vinculin (VCL) and four and a half LIM domains 3 (FHL3) protein were from Sigma. The anti-mouse IgG-HRP, anti-rabbit IgG-HRP, anti-sarcalumenin (SRL) and anti-β-actin (ACTB) were from Santa-Cruz biotechnology (Heidelberg, Germany). The polyclonal antibodies against glycerol-3-phosphate dehydrogenase [NAD+] (GPD1), myozenin 1 (MYOZ1), annexin A1 (ANXA1), annexin A5 (ANXA5), glutathione S-transferase omega-1 (GSTO1), α-crystallin B chain (HSPB5), heat shock protein HSP 90-beta (HSPC3), aldehyde dehydrogenase (ALDH2), NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2), cytochrome b-c1 complex subunit Rieske (UQCRFS1), beta-enolase (ENO3), heat shock 70 kDa protein 1A/1B (HSPA1A) and transitional endoplasmic reticulum ATPase (or valosin-containing protein, VCP) were purchased from Euromedex (Genetex, Souffelweyersheim, France).

Protein extraction. Protein extracts were prepared for each subject, and each individual was assessed separately. For total muscle extracts, biopsies of *vastus lateralis* muscle were from eleven post-menopausal women divided in two groups: adult control (n = 5) and aged (n = 7). Muscle aliquots were homozenized (40mg/mL) in a solubilisation buffer containing 8.3 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (v/v) dithiothreitol, and 2% (v/v) IPG buffer pH 3-10 using a TissueRuptor (Qiagen, Courtaboeuf, France), shaken for 30 min on ice and centrifuged for 30 min at 10,000 X *g*. The supernatants were aliquoted and stored at -20°C until analysis. Protein concentration, determined using the Bradford assay system (Bio-Rad), was 7.2 ± 1.1 mg/mL and 5.5 ± 1.2 mg/ml for the adult and old group, respectively.

Low ionic strength (LIS) extracts were prepared from muscle biopsies of twelve post-menopausal women, either adult (n = 6) or aged (n = 6), according to Sayd et al., 2006 [19]. Briefly, frozen muscle was homogenized using a TissueRuptor in 40 mM Tris (pH 7.0), 2 mM EDTA, and protease inhibitors cocktail (Sigma). After centrifugation at 4°C for 10 min at 10,000 X *g*, the supernatant, referred to as LIS extract, was stored at -80°C. Protein concentration was 9.3 ± 2.2 mg/ml and 9.4 ± 1.6 mg/mL for the adult and old group, respectively.

Two-dimensional gel electrophoresis (2DGE). For total muscle extracts, 700 μg protein were separated per gel using IPG strips of three different pH ranges (pH 3.0-5.6, pH 5.3-6.5 and pH 5-8) for each individual. For isoelectrofocusing, samples were diluted with rehydration buffer containing 8.3 M urea, 1 M thiourea, 2% (w/v) CHAPS, 0.28% (v/v) dithiothreitol, 2% (v/v) IPG buffer (pH 3-10), and 0.01% (w/v) Coomassie Brilliant blue R-250. The IPG strips were passively rehydrated with 330 µl of this solution for 16 h under mineral oil in the PROTEAN IEF Cell system (Bio-Rad) at 20°C, and actively rehydrated using Electrode Wicks loaded onto IPG strips for 6 h at 50 V. During active rehydration, Electrode Wicks were changed every 2 h. Isolectrofocusing was then performed at 0.05 mA per IPG strip at 50 V for 2 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 8000 V for 6 h and finally 8000 V to achieve 46,000 Vh.

For LIS extracts, gels were made in triplicate for each individual and 700 μg protein was analyzed per gel. The IPG strips (pH 5-8) were passively and actively rehydrated as described above and isolectrofocusing was performed at 0.05 mA per IPG strip at 50 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 1000 V for 1 h, 8000 V for 7 h and finally 8000 V to achieve 60,000 Vh.

The strips were then equilibrated twice for 15 min with gentle shaking in equilibration buffer containing 6 M urea, 50 mM Tris-HCl buffer (pH 8.8), 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate. Dithiothreitol (1% w/v) was added to the first, and iodoacetamide (5% w/v) to the second equilibration buffer.

Separation of proteins, according to molecular weight, was carried out using a Protean Plus DodecaCell system (Bio-Rad) on homogenous 20 cm polyacrylamide gels (11% T, 2.6% C). The equilibrated strips were sealed to the top of the horizontal gel with agarose and subjected to 50 V for 1 h followed by 9 mA per gel until the blue dye reached the bottom of the gel.

Visualization of proteins and image analysis. 2DGE were fixed overnight in a solution containing 30% (v/v) ethanol and 2% (v/v) orthophosphoric acid, washed twice for 30 min in 2% (v/v) orthophosphoric acid and then transferred to a solution containing 18% (v/v) ethanol, 2% (v/v) orthophosphoric acid and 15% (v/v) ammonium sulphate for 30 min. The gels were stained for 72 h with 0.06% (w/v) Coomassie Blue G-250 added to this last solution. Gels were scanned using the ImageScanner and LabScan-v.5 software (Amersham Bioscience) and protein spots were analyzed and matched between all gels using Progenesis SameSpot software (Non Linear Dynamics, Newcastle upon Tyne, UK). Proteins with significant changed abundance were picked for tryptic digestion from gels.

Protein identification by mass spectrometry. Excised protein spots from 2DGE were distained with 25 mM ammonium bicarbonate, 5% (v/v) acetonitrile for 30 min and twice in 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile for 30 min each. Protein spots were then dehydrated using 100% acetonitrile for 10 min. Proteins were digested overnight at 37°C using 10 ng/µl of sequence grade-modified trypsin in 25 mM ammonium bicarbonate. Peptide extraction was optimised by adding 100% acetonitrile, followed by 15 min of sonication.

For LC-MS/MS mass spectrometry analysis, peptides mixtures were analysed by on-line nanoflow liquid chromatography using the Ultimate 3000 RSLC (Dionex, Voisins le Bretonneux, France) with 15 cm nanocapillary columns of an internal diameter of 75 µl (Acclaim Pep Map RSLC, Dionex) [20]. The gradient consisted of 4–50% (v/v) acetonitrile in 0.5% (v/v) formic acid at a flow rate of 300 nl/min for 30 min. The eluate (6 µl) was electrosprayed into an LTQVelos (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source. The LTQVelos was operated in a CID top 10 mode (1 full scan MS and the 10 major peaks in the full scan are selected for MS/MS). Raw files were processed using version 1.2 of Thermo Proteome Discoverer. For protein identification, the NCBInr suscrofa protein database (41000 seq) was combined with the sequences of human keratin contaminants.

Peptide mass tolerance was set to 1.5 Da and fragment mass tolerance was set to 0.8 Da. Two missed cleavages were allowed. Protein identification was validated when at least three peptides originating from one protein showed significant identification Mascot scores ($P < 0.05$). In the present study we considered that proteins validated for the whole analysed spots were really present on the basis of score, peptide number related to each protein, and MW agreements.

Immunoblotting. For Western-Blot analysis, total protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred to Hybon^{TM}-P membrane and probed with anti-VCL, anti-FHL3, anti-SRL, anti-MYOZ1, anti-ANXA1, anti-ANXA5, anti-GSTO1, anti-ENO3, anti-GPD1, anti-UQCRFS1, anti-NDUFS2, anti-ALDH2, anti-HSPB5, anti-HSPA1A, anti-HSPC3, or anti-VCP. Primary antibodies were resolved with corresponding horseradish peroxidase-linked goat anti-mouse or anti-rabbit secondary antibodies, and immunoreactive proteins were detected using enhanced chemiluminescence and a Charge Coupled Device camera (GBOX, Syngene, Cambridge, UK). Each blot was dehybridized using 1X ReBlot Plus Strong antibody stripping solution and probed with anti-ACTB for normalization.

Statistical analysis. Values are means $\pm$ SE. Age effect was evaluated using an unpaired Student's t-test procedure with significance set at $P < 0.05$.

Results and Discussion

Differentially expressed proteins during aging

In order to evaluate age-dependent alterations in the skeletal muscle proteome after the fifth decade of life, protein extracts from muscle of adult versus old post-menopausal women were resolved by 2DGE. Gels with overlapping range of IPGs were used to improve the separation of total muscle extracts. 2DGE with medium range IPGs (pH 5-8) revealed 839 protein spots that were matched between all individuals. Among these protein spots, 53 were found to be differentially expressed between adult and old women, and 36 were identified by LC-MS/MS, corresponding to 29 different proteins (Figure 1A). 2DGE with acidic IPGs (pH 3.0-5.6) distinguished 202 matched protein spots, and among them 7 were differentially expressed and identified as 7 different proteins (Figure 1B). Narrow IPGs were also used to achieve optimal resolution in the pH 5.3-6.5 range, and this further revealed 179 matched spots. Among these 179 spots, statistical analysis revealed 3 differentially expressed spots which were identified by LC-MS/MS as 3 different proteins (Figure 1C).

To improve the detection of low abundance proteins, proteins from LIS extracts were also investigated by 2DGE. Thirty-six gels with medium range IPGs (pH 5-8) were analyzed and 699 protein spots were matched between all individuals. Statistical analysis revealed that 86 spots were differentially expressed between adult and old women. Among them, 59 were identified by LC-MS/MS, and they corresponded to 41 different proteins (Figure 2). Targeting the LIS sub-proteome improves the analysis of the muscle proteome, as most (35 out of 41) differentially expressed LIS proteins were not detected in total muscle extracts.

Overall in the present study, 1919 protein spots were matched between all individuals, 106 were differentially expressed and identified, and they corresponded to 73 different proteins. Table 1 summarizes the main properties of the proteins differentially regulated in skeletal muscle between adult and old post-menopausal women.

Perturbations of the myofilament network and cytoskeleton with aging

Sarcomeric proteins

Muscle contraction is generated by an interaction between the molecular motor myosin and filamentous actin. Myosin is a hexameric protein that consists of two heavy chain subunits, two alkali light chain subunits and two regulatory light chain subunits. Myosin light chains typically present various isoforms, and in our study, muscle aging was associated with higher level of one myosin light chain 1/3 skeletal muscle isoform (MYL1, spot 1882) and lower level of one myosin regulatory light chain 2 ventricular/cardiac muscle isoform (MYL2, spot 2032). Perturbations in myofibrillar contractile proteins were confirmed by the up-regulation of one isoform of fast troponin T (TNNT3, spot 1123) which is a major regulator of the thin filament. Troponin T directly interacts with key components in the thin filaments regulatory system to

mediate the activation and force development of actomyosin contractile units [21]. Age-related changes in fibers expressing various myosin light chain isoforms were previously described for MYL1, MYL2 and TNNT3 [11, 13, 16]; however, these changes were manifested differentially in distinct muscles. In our study, there was no clear evidence for a fast-to-slow transition on the unique basis of the differential expression of myosin light chain and troponin isoforms.

Interestingly, the old skeletal muscle exhibited also altered levels of several fragments of sarcomeric proteins. Thus, there were higher levels of N-terminal (spots 1333 and 1415) and C-terminal (spot 1417) fragments of skeletal α-actin (ACTA1), and higher levels of C-terminal fragments (spots 554 and 570) of myosin-1 (MYH1), the type IIX adult fast myosin heavy chain. The old muscle also exhibited lower level of a C-terminal fragment of titin (TTN, spot 1734), when compared to adult muscle. Such fragments were not observed for non-sarcomeric proteins, and may indicate perturbations in muscle proteolytic pathways.

Myofilament regulating proteins

A feature of muscle aging was also the differential expression of several proteins regulating sarcomeres. Aging was thus associated with an increased expression of the ankyrin repeat domain-containing protein 2 (ANKRD2, spot 1361). ANKRD2 is a member of the mechano-sensing proteins that link myofibrillar stress response to muscle gene expression [22, 23]. ANKRD2 interacts both with I-band sarcomeric proteins and with nuclear transcription factors [24]. This stretch-response protein is preferentially expressed in slow type-I fibers, and is induced by denervation, which is consistent with neuronal remodeling in the old muscle [25].

Many extracellular signals elicit changes in the actin filament. One family of proteins that plays a role in actin remodeling in response to cellular signals is the cyclase-associated proteins. In the present study, adenylyl cyclase-associated protein 2 (CAP2, spot 817) is significantly decreased in the old muscle. In skeletal muscle, CAP2 is an M-band protein implicated in regulating actin filament dynamics [26] and its ablation leads to disarray of sarcomere [27].

Cytoskeletal proteins

The integrity of muscle fibers depends on cytoskeletal components, which align sarcomeres and anchor them across the sarcolemma to the basement membrane [28]. Two isoforms of vinculin (VCL, spots 522 and 526) were enhanced during aging. VCL localizes to adhesion junctions and is a central component of muscle costameres [29, 30]. VCL is placed between the integrin-talin complex and the actin cytoskeleton and is a major candidate for transduction of force during the contractile cycle. Besides binding to actin, VCL interacts with signaling networks and is important for signal transduction between the extracellular matrix and the cytoskeleton [31].

Finally, the four and a half LIM domain protein 3 (FHL3, spot 1418), which is down-regulated in our analysis, is an adaptor protein with numerous interaction partners. In adult fibers, FHL3 interacts with cytoskeletal actin [32] and colocalizes with integrin receptors at the periphery of Z-discs [33]. By binding to integrin and actin, FHL3 might then directly link the

cytoskeleton to the extracellular matrix. FHL3 also plays a role in myogenic progenitor cells (satellite cells), where it localizes to the nucleus and has been implicated in the regulation of proliferation [34] and differentiation [35].

ANKRD2, CAP2, VCL and FHL3 have not been previously identified by proteomic surveys of human skeletal muscle aging [8, 16, 17]. Western blotting experiments performed with total muscle extracts confirmed the differential expressions that we observed for VCL and FHL3 in LIS extracts (Figure 3A and B).

In all, the modifications that we report for sarcomeric actomyosin and regulatory proteins are thus likely related to the characteristic disorganization of myofibers in old muscles. Moreover, these changes suggest an influence of aging process on the maintenance of the proper organization of sarcolemma in regular structures that are closely linked to costameres, and on the response to extracellular signals. Thus, the age-related changes of skeletal muscles may result in loss of muscle force and contractile speed and contribute to the development of sarcopenia.

Age-related changes in skeletal muscle affect signal transduction

In skeletal muscle, intracellular calcium (Ca^{2+}) is an important secondary messenger for signal transduction and is essential for cellular processes such as excitation-contraction coupling. Action potentials elicit contractions by releasing Ca^{2+} from the sarcoplasmic reticulum (SR) *via* the ryanodine receptors (RyRs). RyRs are modulated directly or indirectly by various ions, small molecules and proteins, including calsequestrin. In this study, proteomics analysis of muscle aging identified an up-regulation of calsequestrin-1 (CASQ1, spot 665), which is a major intra-SR Ca^{2+} buffer that regulates the activity of RyRs [36, 37].

RyR receptors have several potential phosphorylation sites in their cytoplasmic domains and PKA has been shown to phosphorylate RyRs. Our analysis shows an increased level of the PKA type Iα regulatory subunit (PRKAR1A, spot 1179) during aging. Among other substrates PKA can phosphorylate RyR, and PKA-mediated phosphorylation of RyR may result in leaky RyR channels and impaired Ca^{2+} homeostasis [38, 39].

After initiation of muscle contraction by increasing cytoplasmic Ca^{2+}, Ca^{2+} is pumped back to the SR by sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) leading to relaxation. Sarcalumenins are major luminal glycoproteins that codistribute with SERCA and play a role in Ca^{2+} transport and sequestration [40, 41]. There are two sarcalumenin isoforms (SRL, 160-kDa and 53-kDa) that are generated by alternative splicing [40]. In the present study, the 53-kDa isoform (spot 858) was found to be greatly reduced in aged human muscle, and this reduction was confirmed by Western-blotting (Figure 4A). The age-related decrease of sarcalumenin is in agreement with previous studies in rat muscle indicating a shorter half-life of the 53-kDa isoform [42] and a lower level of the 160-kDa isoform [43].

We have also identified an up-regulation of myozenin-1 (MYOZ1, also termed calsarcin-2, spot 3260) which is expressed in fast-twitch fibers of skeletal muscle and modulates the function

and substrate specificity of calcineurin, a Ca^{2+}/calmodulin-dependent serine-threonine phosphatase that plays an important role in transducing calcium-dependent signals [44]. This increase of MYOZ1 was also confirmed by Western-blotting (Figure 4B)

In skeletal muscle, numerous proteins can bind Ca^{2+}, and muscle aging was further associated with higher level of three members of the annexin family which undergo Ca^{2+}-dependent binding to the cellular membranes. We identified annexin A1 (ANXA1, spot 1494), A5 (ANXA5, spot 3701) and A7 (ANXA7, spot 758). Annexins have been involved in a broad range of molecular and cellular processes. Noteworthy ANXA1, also called lipocortin I, may contribute to the regeneration of skeletal muscle tissue by modulating migration [45] and fusion [46] of satellite cells. ANXA1 is overexpressed in different muscular dystrophies [47] and can further participate in sarcolemmal and T-tubular repair processes [48]. ANXA5 also promotes membrane repair by self-assembling into two-dimensional arrays on membranes [49]. ANXA7, or Synexin, was originally described as a protein that provokes fusion of lipid vesicules [50]. More recently, ANXA7 was identified as an essential protein for autophagy induction by modulating the intracellular Ca^{2+} concentration [51]. Western-blotting experiments confirmed the overexpression of ANXA1 and ANXA5 (Figure 4C and D) in total extracts of old muscle, compared to adult muscle.

Glutathione S-transferase omega-1 (GSTO1) is distinguished from the other glutathione S-transferase family members by a different active center amino acid residue, which results in loss of prototypical glutathione conjugating activity [52]. Instead, human GSTO1 is reported to potentiate skeletal muscle ryanodine receptor (RyR) 1 [53]. The age-related down-regulation of GSTO1 (spot 1673) was confirmed by Western-Blot (Figure 4E), and may thereby be involved in the impairment of Ca^{2+} homeostasis.

Most of these signal transduction proteins (CASQ1, PRKAR1A, MYOZ1, ANXA1 and ANXA7) have never been reported in previous proteomic studies of muscle aging. Overall, these findings suggest significant alterations in Ca^{2+} signaling which may be important for the age-related modifications in muscle contractile properties.

Perturbations in the energy metabolism of old muscle

Disturbance in energy metabolism is another characteristic feature of old muscles. Decreased activities of glycolytic enzymes were previously reported in rat [11] and in human [16] skeletal muscles, whereas expression of mitochondrial enzymes was more controversial in the literature [8, 16]. Our study points to an age-associated decline in key enzymes of the glycolytic, Krebs cycle and oxidative phosphorylation pathways.

Cytoplasmic energy metabolism.

In the current study, perturbations in the energy metabolism of old muscle were indicated by the down-regulation of five isoforms of monomeric creatine kinase (CKM) (spots 1261, 1266, 1276, 1289 and 3617). SDS-stable dimeric and trimeric forms of CKM were previously

described in mice muscle [54], and we also observed age-related decreases in dimeric (spots 330 and 332) and trimeric (spot 516) forms of CKM. Creatine/phosphocreatine is central to maintain energetic homeostasis as it connects intracellular sites of energy demand with sites of ATP production. At these sites, CKM catalyses the transphosphorylation between phosphocreatine and ADP. In sarcomeric M-line, CKM interacts with myomesin and supplies ATP for the actomyosin contractile unit.

The first step of glycogenolysis pathway is the production of glucose-6-phosphate by glycogen phosphorylase (PYGM), which catalyzes the phosphorolytic cleavage of a glucosyl residue from the glycogen polymer. Four isoforms (spots 289, 659, 676 and 696) of PYGM increased, and one (spot 391) decreased with aging. The resulting glucose 1-phosphate molecule is converted by phosphoglucomutase to glucose 6-phosphate. Proteomic analysis revealed a decreased expression of phosphoglucomutase-1 (PGM1, spot 998) in elderly. Several glycolytic enzymes were also down-regulated with aging. Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. In post-menopausal women muscle, five isoforms of the muscle specific β-enolase (ENO3, spots 1209, 1210, 1213, 1215 and 1236) selectively decreased during aging. We also detected lower levels of L-lactate dehydrogenase β (LDHB, spot 1448) which catalyzes the inter-conversion of pyruvate (the final product of glycolysis) and lactate with concomitant inter-conversion of NADH and NAD^+. Finally, down-regulations of glycerol-3-phosphate dehydrogenase (GPD1, spot 1874) and glycerol-3-phosphate dehydrogenase 1-like protein (GPD1L, spot 3459) were observed. Cytosolic GPD1, together with its mitochondrial isoform, constitutes the GPD1 shuttle, which is essential for mitochondrial oxidation of glycolytic NADH. Although less active than its GPD1 counterpart, GPD1L exhibits dehydrogenase activity, and is also implicated in the regulation of hypoxia [55]. Age-related decline in cytoplasmic glycerol-3-phosphate dehydrogenase may indicate reduced mitochondrial oxidation of cytosolic NADH in old muscle.

Two isoforms of triosephosphate isomerase (TPIS, spots 3345 and 3511), that catalyzes the isomerization of the dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate, were more abundant in old women. TPIS ensures that DHAP produced by aldolase is further metabolized by the glycolytic enzymes. Impairment of TPIS may result in chemical conversion of DHAP into toxic methylglyoxal [56] promoting the formation of advanced glycation end-products. In the old muscle, increased levels of TPIS may then represent a compensatory adaptation to avoid excessive formation of toxic products [57].

Mitochondrial energy metabolism.

Alterations in mitochondrial Krebs cycle were revealed by the age-dependent reduction in two subunits of the pyruvate dehydrogenase, pyruvate dehydrogenase E1 component subunit β (PDHB, spot 1196) and dihydrolipoyl dehydrogenase (DLD, spot 769), and by the reduction in aconitate hydratase (ACO2, spot 577) and furamate hydratase (FH, spot 1229), two enzymes that catalyze the isomerization of citrate to isocitrate and the hydration of furamate to malate, respectively. Moreover, alterations in oxidative phosphorylation were also revealed by lower

levels of three components of the respiratory chain, NADH dehydrogenase iron-sulfur protein 2 of complex I (NDUFS2, spot 984), cytochrome b-c1 complex subunit Rieske of complex III (UQCRFS1, spot 3417) and subunit 5A of cytochrome c oxidase of complex IV (COX5A, spot 2713). Aging was further associated with lower level of ATP synthase subunit β (ATP5B, spot 909). Among these proteins implicated in energy metabolism, three (FH, NDUFS2 and UQCRFS1) have never been reported in previous muscle aging studies. Western blotting experiments confirmed alterations in glycolytic and oxidative metabolism as we observed decreased expression of ENO3, GPD1, NDUFS2 and UQCRFS1, in total extracts of old muscle compared to adult muscle (Figure 5). Concomitant decreases of key enzymes of Krebs cycle and major complexes of oxidative phosphorylation provided evidences for alteration of mitochondrial metabolism in the old skeletal muscle.

One component of the respiratory chain complex I, NADH-ubiquinone oxidoreductase (NDUFS1, spot 568), exhibited a higher level in the old muscle. NDUFS1 is a critical caspase substrate, and its cleavage leads to altered mitochondrial transmembrane potential, increased reactive oxygen species production [58], altered lysosomal membrane permeability and release of lysosomal cathepsins [59]. NDUFS1 up-regulation could then represent a counter-mechanism to prevent overproduction of reactive oxygen species and excessive apoptosis in the old muscle.

Lipid metabolism

In addition to glucose, lipids are another source of energy in skeletal muscle. Several intracellular fatty acid binding proteins (FABPs) have been identified. They have important functions in the transport of intracellular fatty acids by increasing their solubility and have been shown to enhance the transport of fatty acids from the cell membrane to the site of oxidation, i.e. the mitochondria, and to the site of esterification into intramyocellular triglycerol [60]. Muscle aging was associated with the down-regulation of two isoforms of heart FABP (FABP3, spots 3150 and 2371), the major FABP in skeletal muscle, and the up-regulation of the adipocyte FABP (FABP4, spot 3215). FABP3 plays an important, but merely permissive role in fatty acid uptake by skeletal muscles [61]. FABP4 is an adipocyte marker, but is also expressed in muscle fibers [60]. Therefore, age-related up-regulation of FABP4 may relate to increased number of adipocytes, and/or to an increased expression in muscle fibers. Of note, the lipid chaperone FABP4 (also known as aP2) was also identified as a predominant positive regulator of toxic lipid-induced endoplasmic reticulim (ER) stress [62], and FABP4 up-regulation could suggest increased ER stress in the old muscle.

Overall, our proteomic analyses therefore provide strong evidences for altered regulation of energy metabolism in the old skeletal muscle.

Detoxification of cytotoxic products and cytoprotection in the old muscle.

Protection against mitochondrial oxidative stress

Mitochondrial dysfunctions may lead to an excessive production of reactive oxygen species (ROS), and accumulating evidences suggest that oxidative stress underlies the aging process in skeletal muscle [10]. The removal of H_2O_2 in cells is mediated by catalase (CAT), glutathione peroxidase and peroxiredoxin (PRDX) [63]. Herein aging of the human skeletal muscle was associated with lower levels of CAT (spot 1007) and higher levels of PRDX3 (thioredoxin-dependent peroxide reductase, spot 1754). Because CAT is localized exclusively in peroxisomes, its function is limited to the inactivation of H_2O_2 diffusing into these organelles. In contrast, PRDX3 is the only peroxiredoxin restricted to mitochondria, and high levels of PRDX3 may provide a primary line of defence against H_2O_2 over-produced by the respiratory chain in old muscle mitochondria.

A wide range of activities have been reported for protein DJ-1 (PARK7, spot 1858), however, there is consensus that PARK7 is responsive and protective against mitochondrial oxidative stress [64]. PARK7 is an atypical peroxiredoxin-like peroxidase [65]. During oxidative attack, PARK7 is relocalized to mitochondria, has a functional role in scavenging mitochondrial H_2O_2 and decreases mitochondrial fragmentation [66]. Therefore elevated levels of PARK7 in the old muscle may be important in regulating cellular antioxidant capacity.

Detoxification of cytotoxic products

Oxidative stress increases the production of cytotoxic aldehydes, which can react with cellular proteins, nucleic acids and cell membranes. Protection against reactive aldehydes is provided by several families of detoxification enzymes, including aldo-keto reductase (AKR) and aldehyde dehydrogenase (ALDH). Our proteomic analysis provided evidences for perturbated scavenging of reactive aldehyde products in the old muscle, as aldehyde dehydrogenase (ALDH2, spot 1107) and alcohol dehydrogenase (AKR1A1, spot 1351), were up-regulated, while aldose reductase (AKR1B1, spot 3328) and delta-1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1, spot 1011) decreased with aging. Similar regulations were previously reported during aging in rat skeletal muscle for ALDH2 [11] and AKR1B1 [12, 67]. However, no previous study of muscle aging notified the differential expression of AKR1A1 and ALDH4A1.

The mitochondrial enzyme ALDH2 detoxifies aromatic and aliphatic aldehydes (including 4-hydroxy-2-nonenal), which are produced during oxidative stress as a result of lipid peroxidation [68]. The cytosolic oxidoreductase AKR1A1 has broad substrate specificity, and similarly catalyzes the reduction of aliphatic and aromatic aldehydes, ketones, and xenobiotics [69]. The up-regulations of ALDH2 and AKR1A1 thus suggest enhanced scavenging of reactive aldehyde products in the old skeletal muscle. Our Western blot analyses confirmed that muscle aging is associated with higher levels of ALDH2 (Figure 6A).

While ALDH2 and AKR1A1 act principally as detoxification enzymes, AKR1B1 and ALDH4A1 have additional roles besides that of detoxification. Accumulating evidences indeed attribute a significant role to AKR1B1 in transducing cytotoxic signals initiated by inflammatory cytokines [70]. AKR1B1 inhibitors were in fact reported to disrupt signalling cascades leading to

the NFkB activation [71], and reduced levels of AKR1B1 may then be important to limit activation of the NFkB pathway in the old muscle.

ALDH4A1 amino acid sequence diverges from the other ALDH [72] and this enzyme is mostly involved in metabolic regulations [73]. ALDH4A1 is metabolically important, since its substrate (glutamic y-semialdehyde) appears as a common intermediate in the degradative and biosynthetic pathways of the amino acids arginine, citrulline, ornithine and proline and from glutamic acid. The down regulation of ALDH4A1 may thus indicate reduced intermediary metabolism in the old skeletal muscle.

Quality control of cellular proteins

To detect, refold, and eventually eliminate abnormal proteins, cells use quality control mechanisms that buffer protein homeostasis (proteostasis) against cellular stress. The proteomic analysis described here demonstrated the differential regulation of 9 spots identified as heat shock proteins (HSP). All were increased with aging and they encompassed 3 HSP groups in human: HSPA, HSPB and HSPC [74].

The small heat shock proteins (HSPB) are ATP-independent chaperones. HSPBs prevent the aggregation of improperly folded or partially denatured proteins, and are involved in their transfer to the ATP-dependent chaperones or to the protein degradation processes such as proteasomes or autophagosomes [75]. Three HSPBs displayed higher levels in old women compared to adult women: heat shock protein beta-1 (HSPB1 or HSP27, spots 1414, 1762, 1763 and 2375), alpha-crystallin B chain (HSPB5 or CRYAB, spot 2356) and heat shock protein beta-6 (HSPB6 or HSP20, spot 2364). Similar up-regulation of HSPB1, HSPB5 and HSPB6 were previously reported for rat muscle aging [11, 13, 67, 76]. Our Western blot experiments performed with total extracts confirmed that the old human muscle exhibited higher levels of HSPB5 than adult muscle (Figure 6B). In addition to chaperone activities, HSPB1 and HSPB5 have the ability to control the redox status, protect the actin cytoskeleton [77] and inhibit apoptotic cell death [78].

The ATP-dependent chaperones of the HSPA group (former HSP70) are essential for proteostasis as they contribute to the folding and assembly of nascent polypeptides, the transport of proteins across membranes, and the selection of misfolded proteins for degradation [79]. Muscle aging was associated with the up-regulation of two HSPA proteins: the heat shock 70 kDa protein 1A/1B (HSPA1A or HSP70, spot 678), and the mitochondrial stress-70 protein (HSPA9 or GRP75, spot 3332). None of these HSPA were previously reported in proteomic analyses of muscle aging, and our Western blot experiments confirmed the age-related up-regulation of HSPA1A (Figure 6C). HSPA1A is the most abundant inducible cytoprotective HSP70 chaperone. Notably in rodents, overexpression of HSPA1A was repeatedly reported to attenuate muscle atrophy induced by immobilization [80], lengthening contractions [81] or cryo-lesioning [82]. It is therefore likely that the old human muscle may increase HSPA1A levels as a protection against muscle atrophy. HSPA9 is a central component of the mitochondrial protein import motor and it plays a key role in the folding of matrix-localized mitochondrial proteins. HSPA9 is

the only known mitochondrial Hsp70 chaperone, and thus serves as a unique scavenger of toxic protein aggregates in human mitochondria [83].

One HSPC was further found to be up-regulated with aging in woman muscle: the heat shock protein HSP 90-beta (HSPC3 or HSP90AB1, spot 755). HSPC3, which was not notified in previous muscle aging studies, is another important chaperone that functions downstream of HSPA in the ATP-dependent folding and conformational regulation of many client proteins, including protein kinases, steroid receptors, endothelial NO synthase and transcription factors [84]. Most of the clients, with which HSPC3 interacts, are thus involved in signal transduction, making HSPC3a critical factor in cell signalling. Western-Blot experiments confirmed the age-related up-regulation of HSPC3 (Figure 6D).

Finally and in addition to the HSP proteins, the chaperone operating in the endoplasmic reticulum (ER), protein disulfide-isomerase A3 (PDIA3, spot 1081), was also found to increase with human muscle aging, and this observation is in agreement with previous studies in rat [11, 85]. PDIA3 is a multifunctional protein with thiol-protein disulphide oxidoreductase activity, which ensures proper folding of glycoproteins and assembly of major histocompatability complex class I complex [86]. Outside the ER, PDIA3 also functions as a plasma membrane receptor for 1α,25-dihydroxy vitamin D3 [87], which might be important with regard of the implication of Vitamin D in aging {Muir, 2011 #267}.

Cytoprotection in the old skeletal muscle

In agreement with a previous study in rat (07Altun), higher levels of two isoforms of carbonic anhydrase 2 (CA2, spots 812 and 3289) were here identified in senescent human muscle. No consensus has been reached in the literature about carbonic anhydrase 3 (CA3), as both increased [12, 17] and decreased levels [8, 13], were observed in old muscles. Our analyses of muscle aging in women indicated an overexpression of 2 isoforms of CA3 (spots 1752 and 1755) and this was confirmed by our shot-gun study [88]. The various isoforms of CAs play a crucial role in CO2-removal and CO2-provision for metabolic processes, and is central for the acid-base balance and the regulatory processes of ion homeostasis. Numerous proteins are involved in ion homeostasis, including the selenium-binding protein 1 (SELENBP1) of which two isoforms were increased (spots 1119 and 1127) and one isoform was decreased (spot 877) in aging.

Proteolysis

Muscle proteins are continuously turning over, and cells contain multiple proteolytic systems to carry out the degradation process. Several components of the major proteolytic systems, i.e. ubiquitin-proteasome [89], lysosomal autophagy [90, 91] and calpains [92], were in the present study differentially regulated with aging in human skeletal muscle. The old muscle thus exhibited higher levels of the ubiquitin-like modifier activating enzyme 1 (UBA1, spot 3614) and the transitional endoplasmic reticulum ATPase (VCP, spot 3654), while the proteasome subunit

beta type-4 (PSMB4, spot 2410) and the UV excision repair protein RAD23 homolog A (RAD23A, spot 3387) were down-regulated during aging.

The VCP ATPase binds multiple ubiquitin ligases and ubiquitinated proteins and triggers extraction of client proteins from complexes or cellular surfaces, often to facilitate degradation by the proteasome [93]. VCP is thus central for the extraction and degradation of endoplasmic reticulum-associated (ERAD) [94] and mitochondrial membrane misfolded proteins [95]. VCP is also involved in a wide variety of cellular processes, such as DNA repair [96], myofibril biogenesis [97], membrane fusion [98], autophagosome maturation [99] and mitophagy [100]. The up-regulation of VCP in skeletal muscle of old women was confirmed by Western-blot (Figure 7). The scaffold protein RAD23A, which was originally identified as an important factor involved in the recognition of DNA lesions [101], also serves as an ubiquitin receptor and plays a central role in targeting polyubiquitynated proteins, including ERAD substrates, for proteasomal degradation [102].

The ubiquitously micro- and millicalpains are heterodimers that both share the calpain small 1 regulatory subunit (CAPNS1). In our study, muscle aging was associated with higher levels of CAPNS1 (spot 1784), which is essential for micro- and milli-calpains stability and proteolytic activity. Through limited cleavage of defined substrates, ubiquitous calpains regulate a wide spectrum of biological functions, including apoptosis, autophagy [103], DNA [104] and membrane [105] repair, inflammation [106], ECM fibrosis [107] and satellite cell adhesion, migration and fusion [108]. Moreover CAPNS1 has been associated with mammalian longevity [109].

In addition to intracellular proteolysis, proteomics analysis of muscle aging identified an up-regulation of the Xaa-Pro dipeptidase (PEPD, spot 1088), an extracellular proteinase which specifically splits iminodipeptides with C-terminal proline or hydroxyproline. Because of the high level of iminoacids in collagen, PEPD seems to be important for extracellular matrix remodelling [110]. At the extracellular level, we also identified two differentially regulated proteins belonging to the family to serine protease inhibitors (serpins). Thus, aging was associated with higher levels of the alpha-1 anti-trysin (SERPINA1, spot 771) and with lower levels of the anti-thrombin III (SERPINC1, spot 1014).

With the exception of VCP, none of these proteolysis potential biomarkers has previously been mentioned in any proteomic study of muscle aging.

Serum and transport proteins

Several spots were identified as differentially expressed plasma transport proteins. A number of these proteins were found decreased in aged muscle, including vitamin D binding protein (GC, spot 1070), transthyretin (TTR, spot 2892) and apolipoprotein A-I (APOA1, spots 1847, 1855 and 3624). Vitamin D binding protein is a major plasma transport protein for vitamin D, while TTR transports thyroxine (T4) and retinol (vitamin A) through the association with retinol-binding protein. As a major component of the high density lipoprotein complex, APOA1 helps to clear fats, including cholesterol, from white blood cells within artery walls. APOA1 is

also partly localized on skeletal muscle lipid droplets [111]. In contrast, serotransferrin (TF, spot 2892), which controls level of iron and stress by increasing iron uptake, was up-regulated in aged muscle.

Other serum proteins were differentially expressed during aging, as a decrease of two isoforms of fibrinogen gamma chain (FGG, spot 917 and 919) and Ig alpha-1 chain C region (IGHA1, spot 424), and an increase of haptoglobin (HP, spot 3276). Altered levels of serum proteins may be related to impaired blood-flow distribution that has been reported in aged muscle fibers [112].

Miscellaneous

Spot 3270, which is decreased 1.4-fold, was identified as elongation factor Tu (TUFM) that mediates the entry of the aminoacyl tRNA into a free site of the ribosome. Three isoforms of the ES1 protein homolog (C21orf33, spots 1429, 1430 and 1838) was also increased in aged muscle. C21orf33 (also known as KNP1) is ubiquitously expressed but strongly so in heart and skeletal muscle and potential mitochondrial targeting signals were found in this protein {Scott, 1997 #265}, but its actual physiologic function is not known. Cytosolic 5'-nucleotidase 3 (NT5C3A, spot 1177) mainly catalyzes the dephosphorylation of pyrimidine nucleoside monophosphates, and therefore plays an important role in both endogenous nucleoside and nucleotide pool balance [113]. The age-related down-regulation of NT5C3A (-1.6 fold) that we observe in senescent women was not previously reported in the literature. Finally, spot 1429 decreased 1.2-fold and is identified as APOBEC2 that belongs to the cytidine deaminase superfamily and mediates editing of mRNA. Interestingly, APOBEC2 deficiency in mice has recently been associated with a shift to a slow-fiber type in muscle, and with diminished body mass and mild myopathy {Sato, 2010 #263}.

To conclude, our proteomics analyses lead to the identification of numerous proteins with a dysregulated expression in the old skeletal muscle. All these proteins have been classified into seven groups, associated with myofilaments and cytoskeleton, energy metabolism, detoxification, cytoprotection, signal transduction and proteolysis (Figure 8). Many of the candidate proteins identified herein by differential proteomics were previously unrecognized (41 out of 73) in previous aging studies of skeletal muscle, in particular proteins implicated in signal transduction or proteolysis. By comparing adult and old post-menopausal women, we have identified a group of proteins in old skeletal muscle, which indicates potential mechanisms of aging and may lead to development of biomarkers of sarcopenia.

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Notes

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Figure legends

Figure 1. Representative 2DGE image obtained from total protein extracts of human vastus lateralis skeletal muscle. 2DGE was performed using a pH range of 5-8 (A), 3-5.6 (B) or 5.6-6.5 (C) in first dimension and SDS-PAGE (11%T) in the second. Protein loading was 700 µg, and the gel was stained using colloidal Coomassie blue G-250. Differentially expressed and identified proteins are marked and spot numbers refer to Table 1.

Figure 2. Representative 2DGE image obtained from sarcoplasmic protein extracts of human vastus lateralis skeletal muscle. 2DGE was performed using a pH range of 5-8 in first dimension and SDS-PAGE (11%T) in the second. Protein loading was 700 µg, and the gel was stained using colloidal Coomassie blue G-250. Differentially expressed and identified proteins are marked and spot numbers refer to Table 1.

Figure 3. Examples of differential expression of cytoskeletal proteins. Representative sections of 2DGE images and representative Western blot for vinculin (VCL) (A) and four and a half LIM domains 3 (FHL3) (B). In each panel, histograms represent normalized volume of protein spot (n=6), and Western blot quantification (n=7) for adult (A) and old post-menopausal women (O). Results are indicated as means ± SE. * $p < 0.05$ indicates significant difference between adult and old women.

Figure 4. Examples of differential expression of proteins implicated in signal transduction. Representative sections of 2DGE images and representative Western blot for sarcalumenin (SRL) (A), myozenin-1 (MYOZ1) (B), annexin A1 (ANXA1) (C), annexin A5 (ANXA5) (D) and glutathione S-transferase omega-1 (GSTO1) (E). In each panel, histograms represent normalized volume of protein spot (n=6), and Western blot quantification (n=7) for adult (A) and old post-menopausal women (O). Results are indicated as means ± SE. * $p < 0.05$ indicates significant difference between adult and old women.

Figure 5. Examples of differential expression of proteins implicated in energy metabolism. Representative sections of 2DGE images and representative Western blot for β-enolase (ENO3) (A), glycerol-3-phosphate dehydrogenase [NAD+] (GPD1) (B), NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2) (C) and cytochrome b-c1 complex subunit Rieske (UQCRFS1) (D). In each panel, histograms represent normalized volume of protein spot (n=6), and Western blot quantification (n=7) for adult (A) and old post-menopausal women (O). Results are indicated as means ± SE. * $p < 0.05$ indicates significant difference between adult and old women.

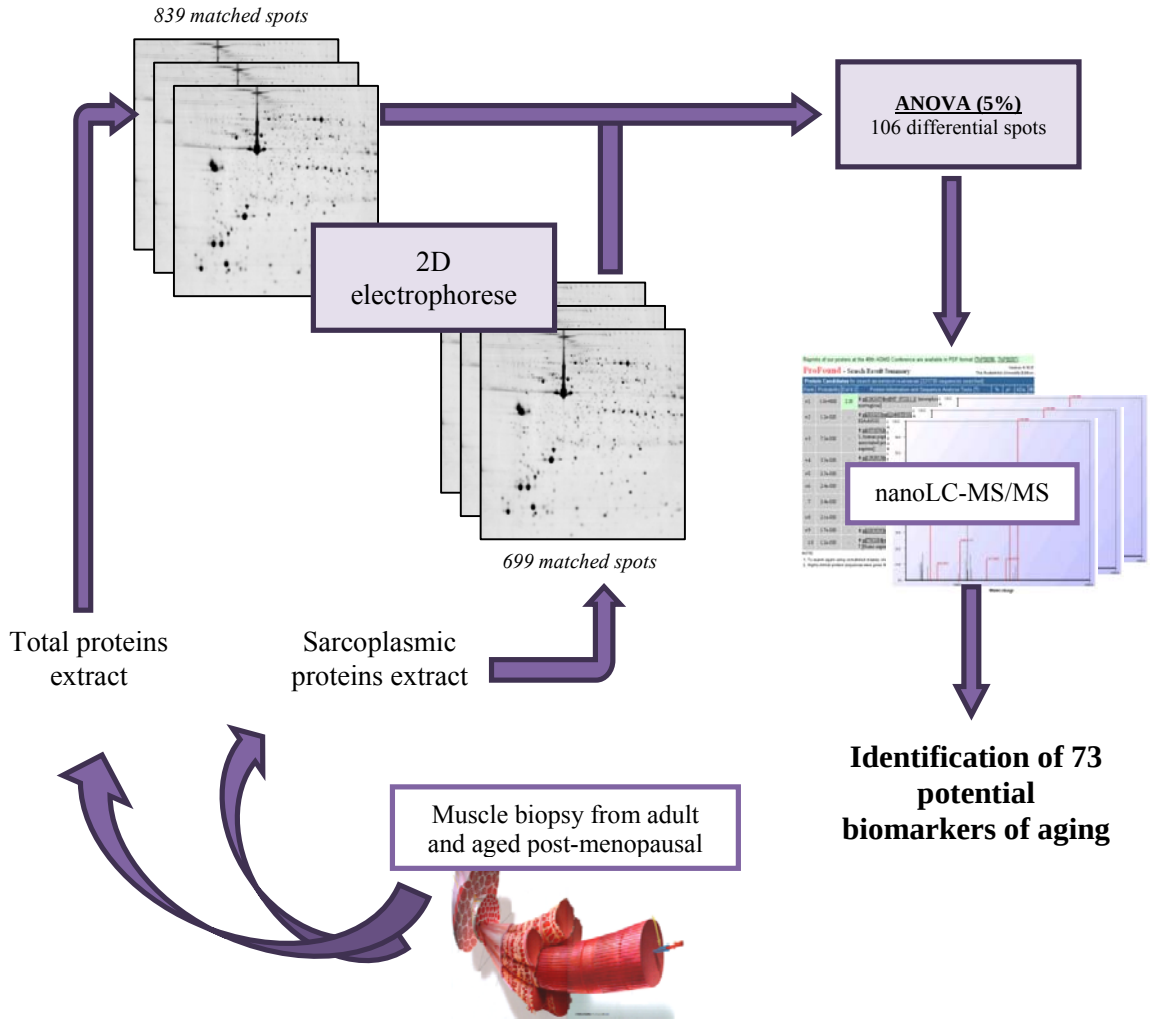
Figure 6. Examples of differential expression of proteins implicated in cytoprotection and cytodetoxification. Representative sections of 2DGE images and representative Western blot for aldehyde dehydrogenase (ALDH2) (A), α-Crystallin B chain (HSPB5) (B), heat shock 70 kDa protein 1A/1B (HSPA1A) (C) and HSP 90-beta (HSPC3) (D). In each panel, histograms represent normalized volume of protein spot (n=6), and Western blot quantification (n=7) for

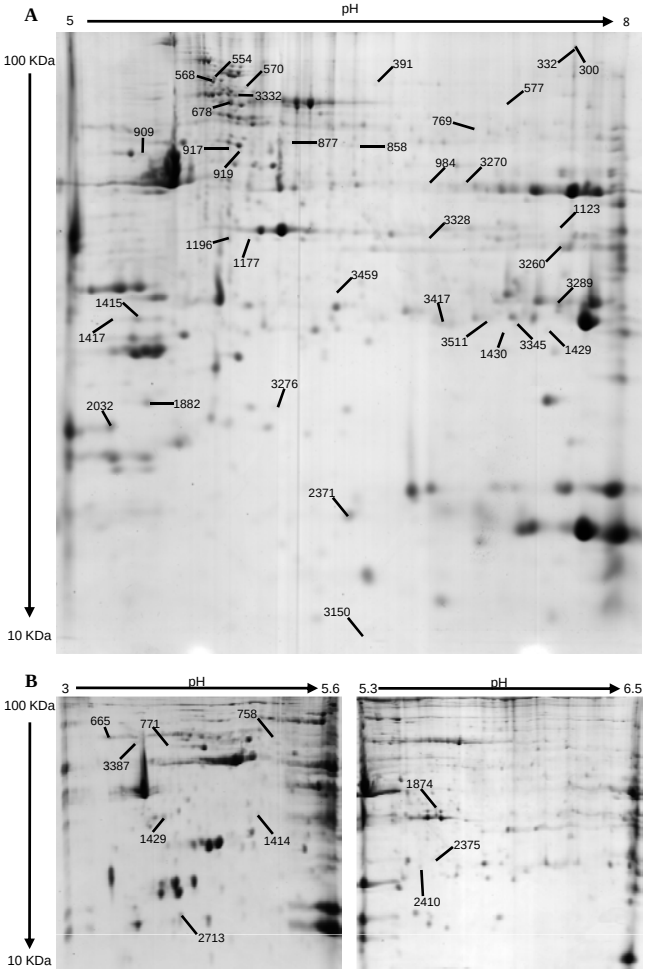
adult (A) and old post-menopausal women (O). Results are indicated as means ± SE. * $p < 0.05$ indicates significant difference between adult and old women.

Figure 7. Example of differential expression of a protein implicated in proteolysis. Representative sections of 2DGE images and representative Western blot for transitional endoplasmic reticulum ATPase (or valosin-containing protein, VCP). In each panel, histograms represent normalized volume of protein spot (n=6), and Western blot quantification (n=7) for adult (A) and old post-menopausal women (O). Results are indicated as means ± SE. * $p < 0.05$ indicates significant difference between adult and old women.

Figure 8. STRING interaction network showing the association between differentially expressed proteins in old post-menopausal women compared to adult women (String database; {Szklarczyk, 2011 #261}). The interaction map was generated using default settings (Medium confidence of 0.4 and 7 criteria for linkage: neighborhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining). The proteins names used in this network are listed in Table 1.

Abstract graphic

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10 KDa
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1123
3328
1196
1177
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1415
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1417
3511
1430
3345
1429
3276
2032
1882
2371
3150
B
3
pH
5.6
5.3
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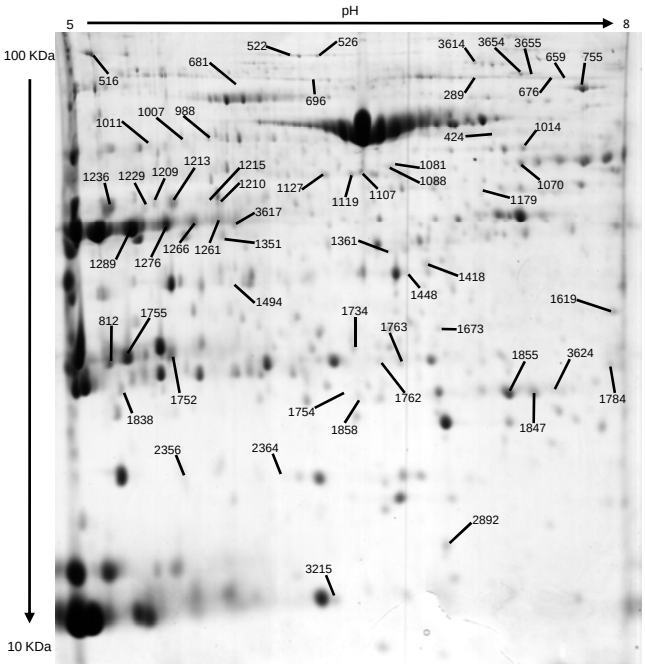
Figure 1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1011
424
1014
1213
1215
1081
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1229
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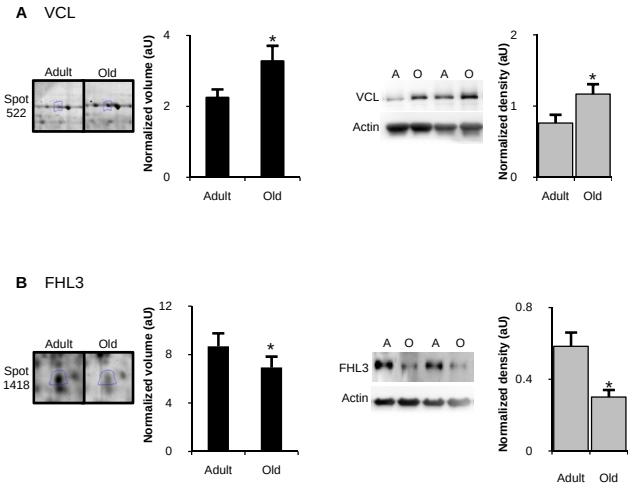
Figure 2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VCL
Actin
Normalized density (aU)
2
1
0
Adult
Old
B FHL3
Adult
Old
Spot 1418
Normalized volume (aU)
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8
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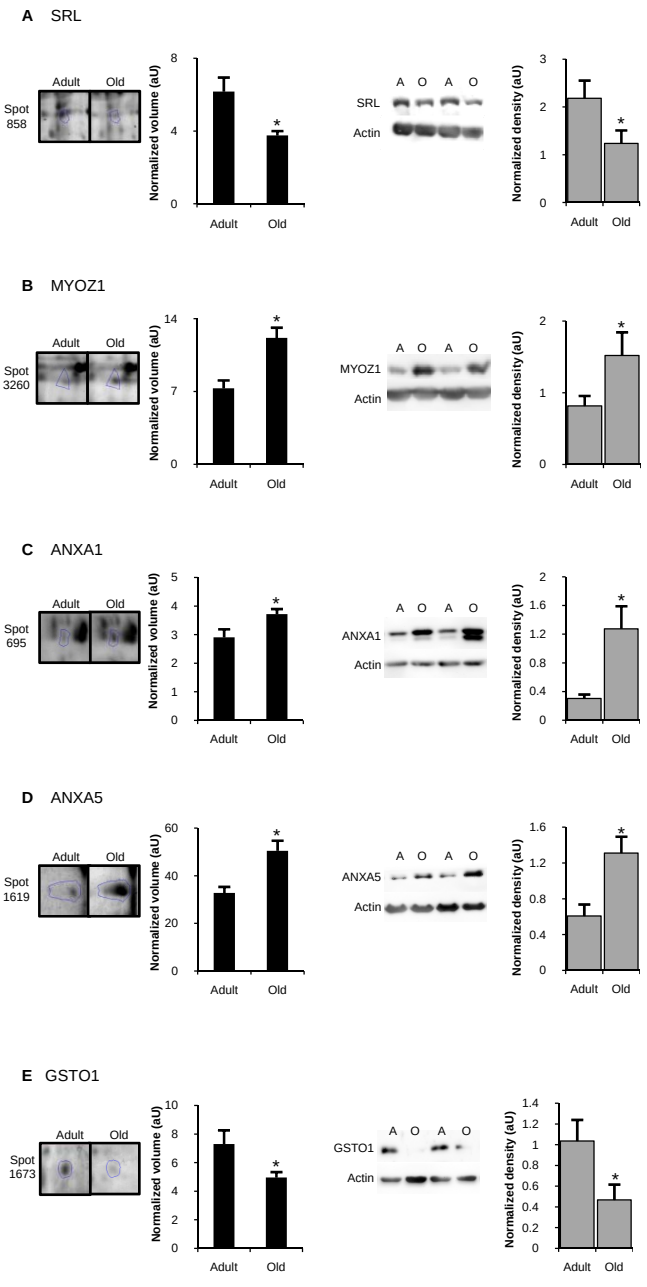
Figure 3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MYOZ1
Actin
Normalized density (aU)
Adult
Old
C ANXA1
Adult
Old
Spot 695
Normalized volume (aU)
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A O A O
ANXA1
Actin
Normalized density (aU)
Adult
Old
D ANXA5
Adult
Old
Spot 1619
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Old
E GSTO1
Adult
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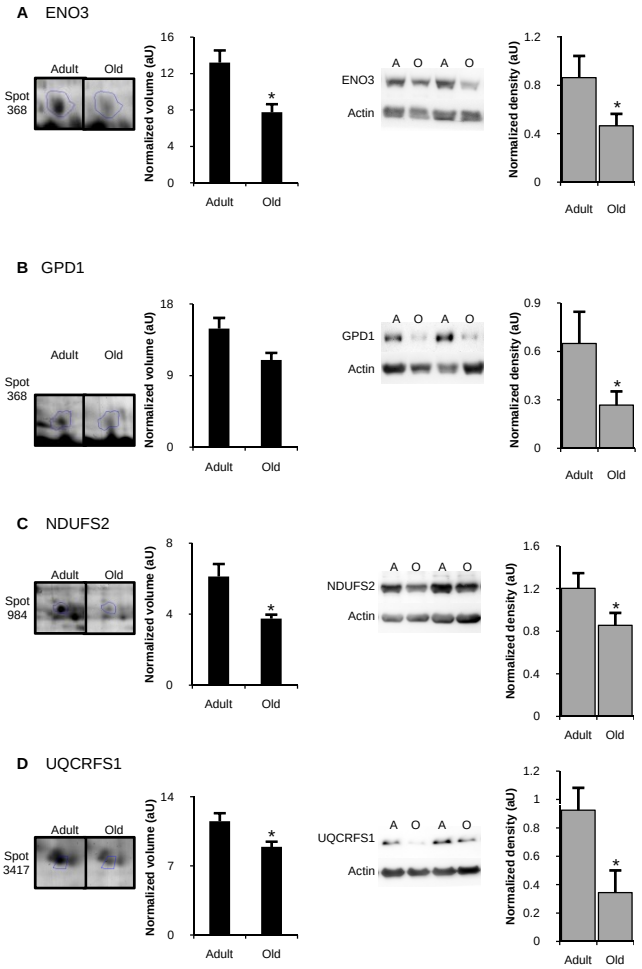
Figure 4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0.4
0
Adult
Old
B GPD1
Adult
Old
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9
0
Adult
Old
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GPD1
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0.3
0
Adult
Old
C NDUFS2
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Old
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1.2
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0.4
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Old
D UQCRFS1
Adult
Old
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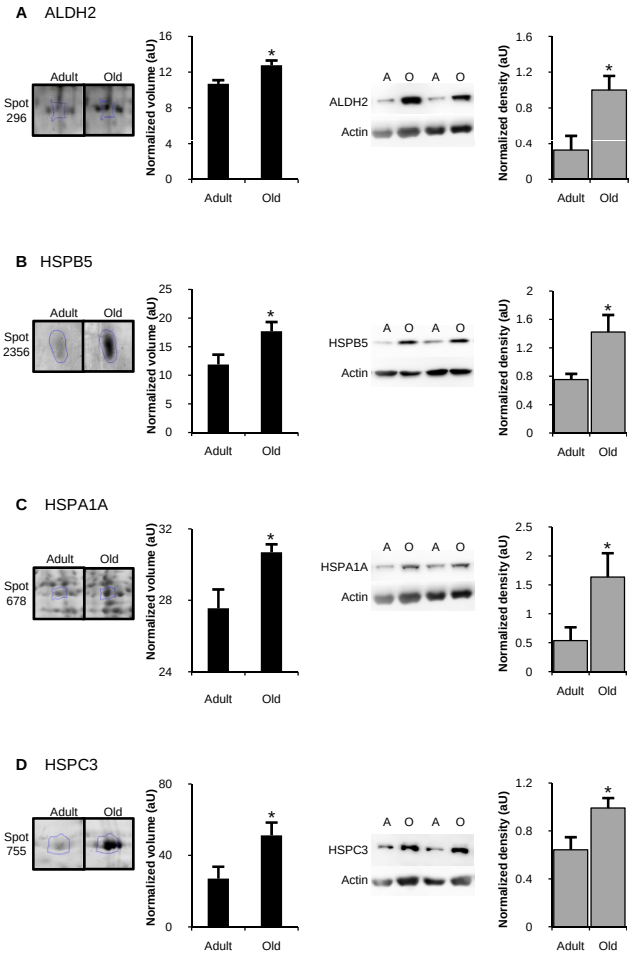
Adult
Old

Figure 5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ALDH2
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Normalized density (aU)
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1.2
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0.4
0
Adult
Old
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Spot 2356
25
20
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10
5
HSPB5
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D HSPC3
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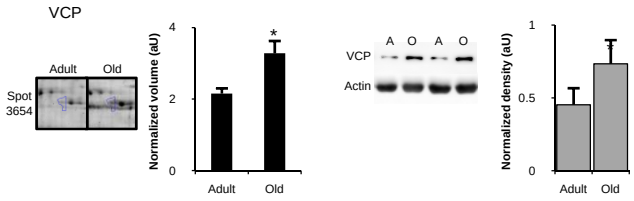
HSPC3

Figure 6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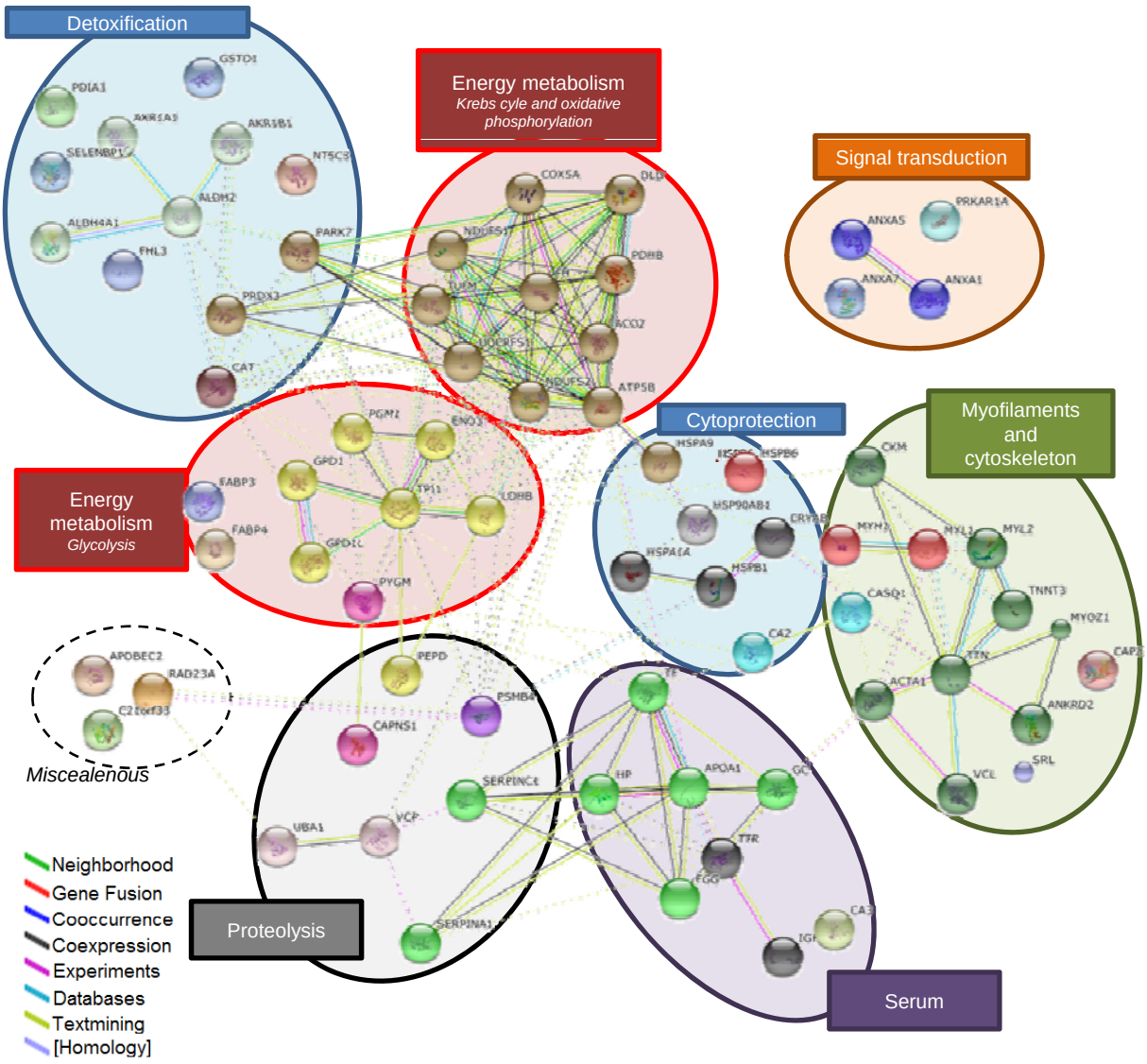
Adult
Old

Figure 7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Gene Fusion
Cooccurrence
Coexpression
Experiments
Databases
Textmining
[Homology]

Figure 8

Publication 2

Laëtitia Théron, Marine Gueugneau, Cécile Coudy, Didier Viala, Astrid Bijlsma, Gillian Butler-Brown, Andrea Maier, Daniel Béchet, Christophe Chambon

"Label-free protein profiling of *vastus lateralis* muscle during human aging"

Molecular & Cellular Proteomics, Doi: 10.1074/mcp.M113.032698

Label-free Quantitative Protein Profiling of *vastus lateralis* Muscle During Human Aging*[S]

AQ: A

Laëtitia Théron‡§, Marine Gueugneau§¶¶, Cécile Coudy§¶¶, Didier Viala‡, Astrid Bijlsma¶, Gillian Butler-Browne‖, Andrea Maier, Daniel Béchet§‡‡, and Christophe Chambon‡§§**

Sarcopenia corresponds to the loss of muscle mass occurring during aging, and is associated with a loss of muscle functionality. Proteomic links the muscle functional changes with protein expression pattern. To better understand the mechanisms involved in muscle aging, we performed a proteomic analysis of *Vastus lateralis* muscle in mature and older women. For this, a shotgun proteomic method was applied to identify soluble proteins in muscle, using a combination of high performance liquid chromatography and mass spectrometry. A label-free protein profiling was then conducted to quantify proteins and compare profiles from mature and older women. This analysis showed that 35 of the 366 identified proteins were linked to aging in muscle. Most of the proteins were under-represented in older compared with mature women. We built a functional interaction network linking the proteins differentially expressed between mature and older women. The results revealed that the main differences between mature and older women were defined by proteins involved in energy metabolism and proteins from the myofilament and cytoskeleton. This is the first time that label-free quantitative proteomics has been applied to study of aging mechanisms in human skeletal muscle. This approach highlights new elements for elucidating the alterations observed during aging and may lead to novel sarcopenia biomarkers. ***Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.032698, 1–12, 2014.***

A gradual degenerative loss of skeletal muscle mass and function is one of the most consistent hallmarks of normal aging. When it reaches defined thresholds, this condition is referred to as sarcopenia (1, 2), and can be associated with disability, poor quality of life, frailty, and increased mortality (3). Aging impacts the morphology, function and biochemical properties of skeletal muscle, but the mechanisms leading to the changes in muscle tissue remain unclear.

Proteomics links the muscle functional changes with the protein expression pattern. Several proteomic approaches have already been used to study sarcopenia. Protein profiling of whole tissue homogenates has been performed using two-dimensional gel electrophoresis (2DGE)[1] and mass spectrometry to identify the proteins differentially expressed during aging in rat (4–6) and human muscle (7, 8). Other studies have focused on specific fractions such as mitochondrial proteins (9), phosphoproteins (10), glycoproteins (11), basic proteins (12), or calpain interacting proteins (13). The few proteomic studies available on human skeletal muscle are mostly based on the 2DGE approach, which implies focusing on a specific pH range (7, 8). Despite its power of high-resolution, 2DGE presents a limited dynamic range and scarcely resolves low abundance regulatory proteins, hydrophobic proteins, and proteins with extreme pI and/or M_r (14). Fn1

To circumvent these limitations, we propose in the present study to apply a label-free protein profiling based on a shotgun proteomics approach. This technique permits to identify proteins in a complex mixture after trypsin hydrolysis, using a combination of high performance liquid chromatography and mass spectrometry. In a shotgun analysis previously performed on whole muscle extracts, the major isoforms of myosin heavy-chain comprise ~42% of the total spectra (15). Because these major isoforms may hamper identification of

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Author contributions: L.T., A.B., G.B., A.M., D.B., and C. Chambon designed research; L.T., M.G., C. Coudy, D.V., and C. Chambon performed research; L.T., M.G., C. Coudy, D.V., D.B., and C. Chambon contributed new reagents or analytic tools; L.T., D.B., and C. Chambon analyzed data; L.T., D.B., and C. Chambon wrote the paper.

[1] The abbreviations used are: 2DGE, two-dimensional gel electrophoresis; LIS, low ionic strength; FDR, false discovery rate; PCA, principal component analysis.

TABLE I

*Physical characteristics of mature (n = 6) and old (n = 4) women. Age, weight (kg), height (cm), and Body Mass Index[1] (BMI; kg/cm) are expressed as mean ± standard deviation and statistical results are indicated (***: p value < 0.01; NS: Non Significant)*

	Mature women $n = 6$	Old women $n = 4$	Significance
Age	53.0 ± 3.5	77.6 ± 2.0	***
Weight, kg	69.3 ± 14.7	74.3 ± 13.0	NS
Height, cm	166.5 ± 8.9	165.3 ± 13.0	NS
BMI[1], kg/cm	25.0 ± 4.9	27.1 ± 3.6	NS

other proteins, we decided to precipitate myofibrils at low ionic strength (16, 17) and to focus on the soluble fraction. In this paper, we present the analytical steps of label-free quantitation, which resulted in the identification and quantitation of 255 muscle proteins common to all ten individuals. The comparison of protein profiling between mature and older women highlighted 35 differentially expressed proteins during aging, 25 proteins that have not previously been related to muscle aging. The functional interactions network linking these proteins showed that the two main biological processes were represented by proteins involved in energy metabolism and contractile proteins.

EXPERIMENTAL PROCEDURES

Subjects—Patients were included in the Rijnland Hospital (Leiderdorp, The Netherlands), and in the Leiden University Medical Center (Leiden, The Netherlands) between June 2010 to September 2012. Exclusion criteria consisted of previous knee or hip surgery (with the exception of arthroscopy), rheumatoid disease, diabetes mellitus, use of oral corticosteroids, and metastasized malignancy. Ten post-menopausal women undergoing hip surgery for hip arthrosis were selected in the present analysis. The mean age was 53.0 ± 3.5 years ($n = 6$) for the mature group, and 77.6 ± 2.0 years ($n = 4$) for the old group. As described in Table I, the mean height, body weight, and body mass index (BMI) were similar between the two groups of women. The study was approved by the medical ethical committees of Leiden University Medical Center and the Rijnland Hospital (P10.060 - HEALTH-2007–2.4.5–10: Understanding and combating age related muscle weakness "MYOAGE"). Written informed consent was obtained from all patients.

The *Vastus lateralis* muscle samples were obtained during surgery, immediately frozen in liquid nitrogen and stored at −80 °C until used.

Protein Extraction—Proteins soluble at low ionic strength (LIS) were extracted from muscle biopsies as described by Sayd *et al.* (18). Frozen samples were homogenized in 40 mM Tris (pH 7.0), 2 mM EDTA, and protease inhibitors mixture using a TissueRuptor (Qiagen, Courtaboeuf, France). After centrifugation at 4 °C for 10 min at 10,000 × *g*, the supernatant, referred to as the LIS extract, was stored at −80 °C.

Protein Separation—Samples were mixed with 1 volume 2% (w/v) SDS, 5% β-mercaptoethanol, 10% glycerol and 62 mM Tris-HCl, pH 6.8 (19), and heated at 95 °C for 5 min. SDS-PAGE (12% acrylamide) was performed using a Mini-Protean II electrophoresis unit (BioRad, Marnes-La-Coquette, France). Samples were loaded at 20 μg protein per lane. To concentrate the samples, gels were run at 100 V until the dye front reached the bottom of the concentration gel. Gels were stained overnight in Coomassie brilliant blue G-250. Excised lanes were reduced in 10 mM dithiotreitol in 50 mM acetonitrile, and alkylated in 55 mM iodoacetamide in 50 mM acetonitrile.

Protein Digestion—Each lane was incubated in 25 mM ammonium bicarbonate with acetonitrile 50/50 (v/v) until destaining. After incubation in 100% acetonitrile, gel pieces were dried in a vacuum SpeedVac. They were further rehydrated with 30 μl of a trypsin solution (10 ng/μl in 25 mM ammonium bicarbonate; V5111, Promega), and finally incubated overnight at 37 °C. Peptide extraction was optimized by adding 24 μl of acetonitrile 100% followed by 10 min of sonication. The trypsin digests were dried in a vacuum SpeedVac and stored at −20 °C in a solution of 2% acetonitrile, 0.05% trifluoroacetic acid before LC-MS/MS analysis.

Nano-LC-MS/MS Analysis—For Nano-LC-ESI-MS/MS analysis, peptides mixtures were analyzed in duplicate by online nanoflow liquid chromatography using the Ultimate 3000 RSLC (Dionex, Voisins le Bretonneux, France) with nanocapillary columns of 25 cm length × 75 μm I.D., 3 μm, 100Å (Acclaim PepMap100 C18, Dionex). The solvent gradient increased linearly from 4% to 50% acetonitrile in 0.5% formic acid at a flow rate of 200 nL/min for 100 min. The elute was then electrosprayed in positive-ion mode at 2.7 kV in a LTQ-VELOS mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source that was operated in a CID top 10 mode (*i.e.* 1 full scan MS and the 10 major peaks in the full scan were selected for MS/MS). Full-enhanced-scan MS spectra were acquired with 1 microscan (*m/z* 400 - 1600). Dynamic exclusion was used with 2 repeat counts, 15 s repeat duration and 45 s exclusion duration. For MS/MS, isolation width for ion precursor was fixed at 2 *m/z*, single charged species were rejected; fragmentation used 37% normalized collision energy as the default activation of 0.25.

Database Search and Protein Identification—Thermo Proteome Discoverer v1.3 was used for raw data file processing, and MASCOT was used for database search (http://www.matrixscience.com). For protein identification, the Uniprot Taxonomy Human (01/10/2012, 84,843 seq) protein database was used. The following parameters were considered for the searches: peptide mass tolerance was set to 1.5 Da, fragment mass tolerance was set to 0.5 Da and a maximum of two missed cleavages was allowed. Variable modifications were methionine oxidation (M) and carbamidomethylation (C) of cysteine. Protein identification was considered valid if at least one peptide with a statistically significant Mascot score assigned it (with Mascot score ≥ 36 for p value < 0.05 with a False Discovery Rate (FDR) at 1%). Identification of proteins based on one peptide was accepted after checking the correct assignment of fragment ion matches (at least three consecutive fragments b/y, match peaks well above the background noise) (supplementary Data). Identifications not satisfying these defined criteria were rejected.

Label-free Quantification—The acquired spectra (Thermo raw files) were loaded into the Progenesis LC-MS software (version 4.1, Nonlinear Dynamics, Newcastle, UK) and label-free quantification was performed. Briefly, for each migration lane from the SDS-PAGE, the profile data of the MS scans as well as MS/MS spectra were transformed to peak lists with Progenesis LC-MS using a proprietary algorithm and then stored in peak lists comprising *m/z* and abundance. One sample was set as the reference, and the retention times of all other samples within the experiment were automatically aligned to create maximal overlay of the two-dimensional feature maps. At this point, features with only one charge, with retention time windows lower than 6 s or with retention time lower than 20 min and higher than 80 min were masked and excluded from further analyses. All remaining features were used to calculate a normalization factor for each sample that corrects for experimental variation. Samples were then allocated to their experimental group (mature *versus* older women). For quantification, all unique validated peptides (with Mascot score ≥

36 for p value < 0.05) of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. Statistical analysis was performed using the "between subject design" and p values were calculated by a repeated measures analysis of variance using the normalized abundances across all runs.

Immunoblot Analysis—Western-blot analyses were performed on whole muscle extracts. Muscle aliquots were lysed in ice-cold buffer containing 8.3 M urea, 2 M thiourea, 2% CHAPS, 1% dithiotreitol and extracts were clarified at 10,000 × g for 30 min. Whole muscle extracts (20 μg proteins) were resolved by SDS-PAGE and electrotransferred to Hybon™-P membranes (Dutscher, Brumath, France). Membranes were probed with anti-transgelin (1:8000; Euromedex, Souffelweyersheim, France), anti-medium-chain specific acyl-CoA dehydrogenase (1:2000; Euromedex), anti-alpha-2-HS glycoprotein (1:3000; Euromedex), anti-aspartate aminotransferase (1:2000; Euromedex), anti-ATP synthase subunit alpha (1:1000; Euromedex), and anti-carbonic anhydrase 3 (1:8000; Euromedex), diluted with TTBS (5% milk in 25 mM Tris (pH 7.8), 150 mM NaCl, 0.1% Tween 20). Primary antibodies were resolved with corresponding horseradish peroxidase-linked secondary antibodies (Luminata, Millipore, Molsheim, France), and immunoreactive proteins were detected using enhanced chemiluminescence and a Charge Coupled Device camera (GBOX, Syngene, Cambridge, UK). For normalization, membranes were dehybridized in 1× Re-blot Plus (Millipore) for 30 min, washed three times 5 min with TTBS, and probed overnight with anti-actin (1:10000) in TTBS. To determine the significance of aging, a Student's t test was used; results are expressed as the mean ± standard deviation.

Functional Correlation and Pathway Analysis—The differentially expressed proteins defined by label-free analysis were classified using the Gene Ontology categories "Cellular Component," "Molecular Function," and "Biological Process." Pathway analysis was performed using the Search Tool for the Retrieval of INteracting Genes (String) 9.0 database (http://string-db.org, (20)). String analysis options were based on "evidence" mode, disconnected nodes were hidden, we did not add or remove any protein partners, and we used clustering by K means to reveal subgrouping within the network.

RESULTS AND DISCUSSION

The goals of this study were to identify and quantify muscle proteins during human aging. For that purpose, we used a proteomic approach combining shotgun methodology and label-free quantitation. We compared the protein profiling of muscle low ionic strength (LIS) extracts between two groups of mature and older women to determine the proteins differentially expressed during muscle aging.

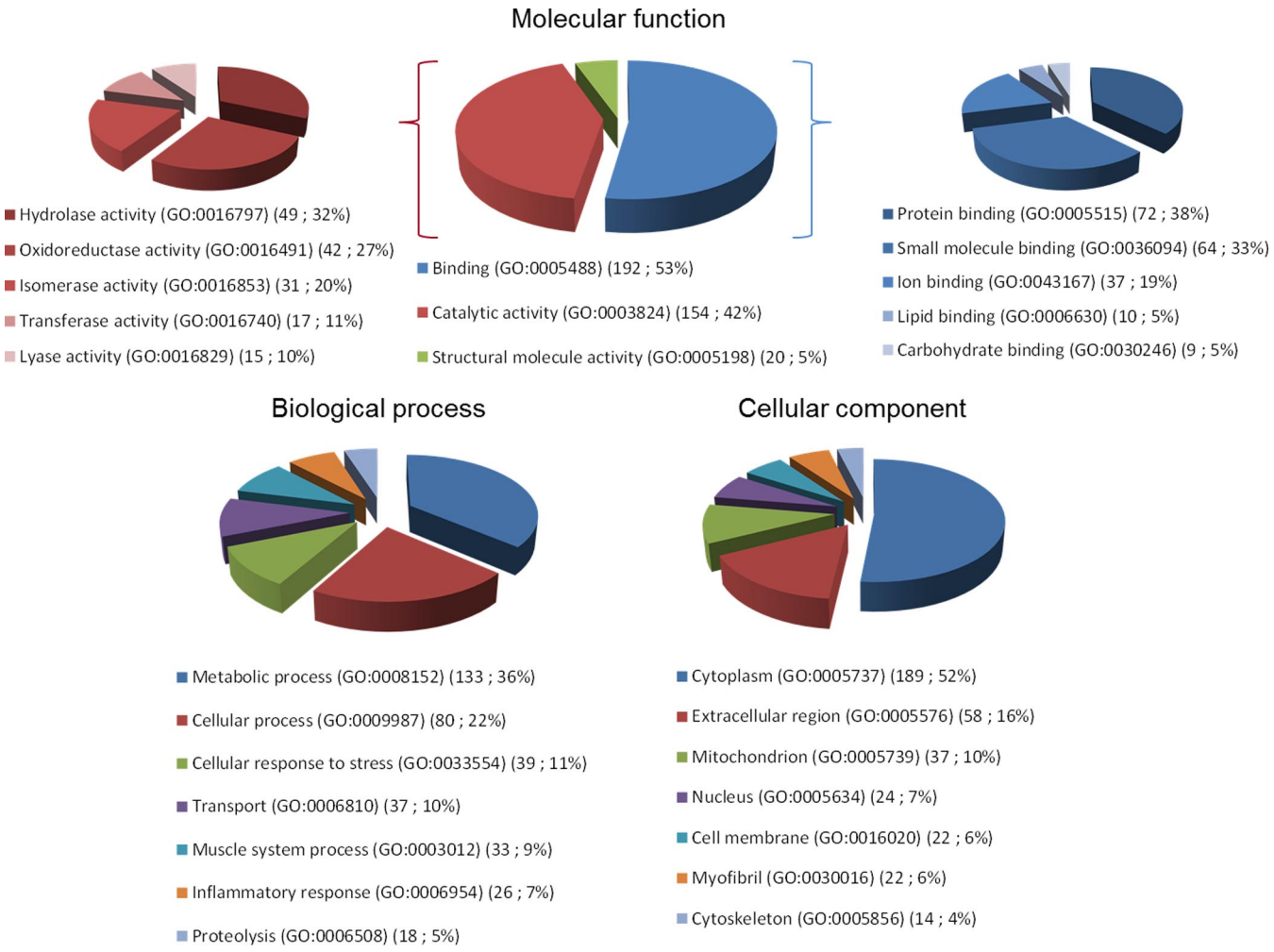
Protein Identification—We performed protein extraction from human *vastus lateralis* muscle to collect the LIS fraction. The amount of proteins extracted was similar between the mature and old women (9.30 ± 2.02 mg/ml and 9.47 ± 1.51 mg/ml respectively), indicating that extractability of LIS fraction was not affected by aging. For each sample, we performed duplicates of LC-MS/MS mass spectrometry analysis. Following the protein digestion of duplicated samples, a total number of 366 proteins were identified (Supplemental Table S1). ZSI

The quantitation analysis by LC-Progenesis was performed on the 255 proteins detected in all 10 samples. All the proteins identified in both mature and older women were classified using the Gene Ontology categories: "Molecular function," "Biological process," and "Cellular component" (Fig. 1). F1 The classification of proteins according to their molecular function showed a repartition between binding activity (53%), catalytic activity (42%), and structural molecule activity (5%). The biological process repartition analysis showed that the proteins were mainly involved in metabolic (36%) and cellular (22%) processes. The metabolic processes include protein, lipid, and carbohydrate metabolisms. The cellular component analysis revealed that the main protein localization was cytoplasmic (52%), which is consistent with the extraction method we used. Moreover, a previous proteomic study performed on human *vastus lateralis* muscle (15), showed that cytoplasmic proteins represented 59% of whole muscle proteome.

Label-free Quantitation of Proteins from LIS Fraction—The label-free quantitation analysis of LIS protein fraction from mature and older women was performed with LC-Progenesis software, using an algorithm based on the pairwise features detection at the LC-MS level (20). The total ion chromatograms of LIS protein fraction from mature (pink) and older (green) women are shown in Fig. 2*A*. A regression analysis F2 based on the liquid chromatography retention time and the m/z of all detected features (example in Fig. 2*B*) was calculated and resulted in an alignment grid that included all detected peptides (Fig. 2*C*). One sample was chosen as the reference and the other samples were aligned using on average 357 and 456 alignment vectors in samples from mature and older women respectively. The overall percentage score for the run alignment was on average 85% showing the quality of alignment. For each aligned peptide, the ion intensity was calculated, so that the relative abundances of the peptide among the mature and older women were determined. The fragmentation spectrum of each quantified peptide was introduced in the analysis, so that the protein identity and abundance were obtained and compared between mature and older women (example in Fig. 2*D*). For each differentially expressed protein quantified from one peptide, we plotted the normalized abundances for all the individuals (supplemental Table S2); for each differentially expressed protein quantified from more than one peptide, the normalized abundances of all the peptides are represented in supplemental Table S3. ZSI ZSI

The results of the label-free quantitation analysis performed on the 255 LIS proteins indicated that 35 were significantly differentially expressed between mature and older women (Table II). Principal component analysis (PCA) was applied to T2 reveal variances or combination of variables among these differentially expressed proteins. The analysis resulted in a good separation between mature and older women according to the principal components one with 42.6% of explained variance (Fig. 3). Statistical analysis revealed that 29 proteins F3 were under-represented and six proteins were over-represented in the older women (Table II).

We performed pathway analysis to determine the functional interactions network linking the differentially expressed pro-



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ZSI FIG. 1. **Identified proteins from LIS fraction of *vastus lateralis* muscle of mature and old women (Supplemental Table 1) analyzed with the PANTHER bioinformatics tool (http://www.pantherdb.org) using the Gene Ontology categories "Molecular function," "Biological Process," and "Cellular Component."** The molecular functions "Catalytic activity" and "Binding" are detailed. For each category, the number of identified proteins and their percentage of the total number of proteins in the pie chart are indicated for each Gene AQ: C Ontology term.

teins between mature and older women. For this, we data mined these findings with the String database (20). This analysis showed that 23 out the 35 proteins were implicated in the F4 resulting network (Fig. 4). To determine the substructure of the network, we applied a K-mean classification that revealed three groups of proteins. The first one (red cluster) was mainly composed by contractile proteins and associated: titin (TTN), ankyrin repeat domain-containing protein 2 (ANKRD2), myosin light chain 1/3 skeletal muscle isoform (MYL1), myosin-1 (MYH1), actin cytoplasmic 1 (ACTB), cofilin-1 (CFL1), and transgelin (TAGLN). The second group (blue cluster) included predominantly proteins involved in energy metabolism: fatty acid binding protein adipocyte (FABP4), aspartate aminotransferase mitochondrial (GOT2), fumarate hydratase mitochondrial (FH), and L-lactate dehydrogenase B chain (LDHB); one protein with a different biological function was related to this cluster: the heat shock protein 70 kDa protein 4 (HSPA4). The third group (yellow cluster) also contained proteins involved in energy metabolism: transaldolase (TALDO1), 6-phosphofructokinase liver type (PFKL), ATP synthase subunit alpha mitochondrial (ATP5A1), ATP synthase subunit beta mitochondrial (ATP5B), medium-chain specific acyl-CoA dehydrogenase mitochondrial (ACADM), carnitine O-acetyltransferase (CRAT), and ADP/ATP translocase 1 (SLC25A4); and other linked proteins: alpha-2-HS-glycoprotein (AHSG), elongation factor 2 (EEF2), and annexin A2 (ANXA2). One more protein was related to this cluster: cAMP-dependent protein kinase type II-alpha regulatory subunit (PRKAR2A). The functional interactions network we built through all the significantly differentially expressed proteins revealed that the main impact of aging on the skeletal muscle LIS proteome was on energy metabolism and contractile properties.

To select proteins as candidates to be validated by Western-blot analysis, we chose to focus on the main biological

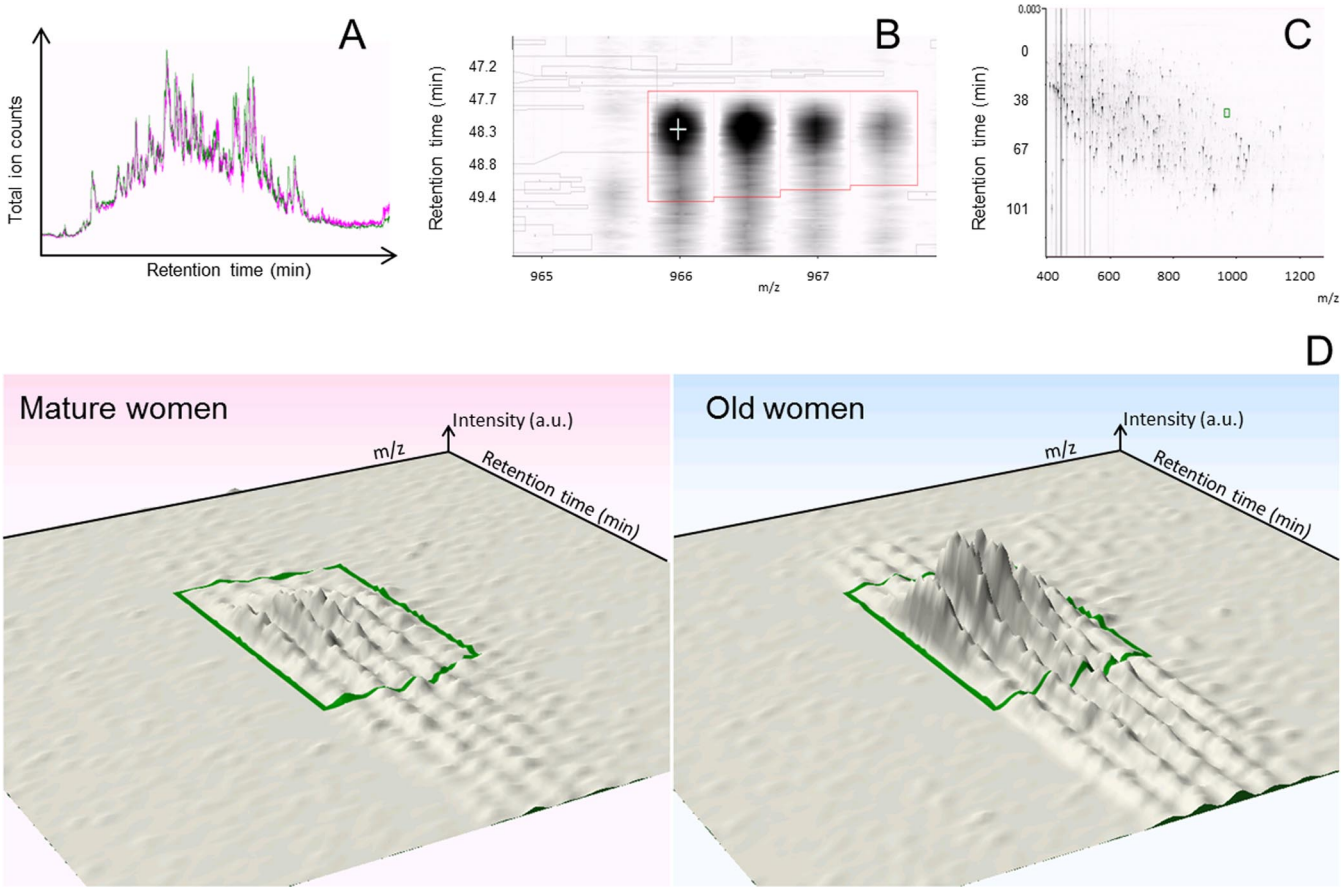

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FIG. 2. **Quantitative analysis with LCProgenesis software.** *A*, Total ion chromatograms of LIS protein fraction from mature (pink) and old (green) women. *B*, Detected feature of carbonic anhydrase 3, according to the liquid chromatography retention time and the *m/z*. *C*, Alignment grid according to the liquid chromatography retention time and the *m/z* of all the detected peptides. *D*, Representation of the abundance of one peptide from carbonic anhydrase 3 protein within mature (pink) and old (blue) women representative samples.

functions, *i.e.* "energy metabolism," "actin myofilament," "detoxification and cytoprotection," and "serum proteins." Thus, we studied transgelin abundance within mature and old women because it was identified and quantified by one peptide; transgelin belongs to the cytoskeleton. Then, we studied medium-chain specific acyl-CoA dehydrogenase, aspartate aminotransferase and ATP synthase subunit alpha abundances within mature and old women. We chose these proteins because their main biological function corresponds to energy metabolism and were all less abundant in older women. We chose carbonic anhydrase 3, which belong to the detoxification and cytoprotection cluster, to confirm its higher abundance in old women because its expression was previously described in literature. We studied alpha-2-HS glycoprotein abundance within mature and old women because it was identified and quantified by one peptide, and it belongs to the serum proteins. Some of these proteins are identified for the first time to be involved in age related sarcopenia. Their validation by Western-blot would confirm their relevance in our biological system. To check that there was no difference in the overall protein expression pattern between individuals used in this study, we performed a control full length protein gel (supplementary Data 2). Statistical results revealed that all of these six candidates proteins were validated by Western-blot analysis (Fig. 5), because the abundances calculated within mature and old women groups were statistically different: aspartate aminotransferase (p value < 0.01), medium-chain specific acyl-CoA dehydrogenase (p value < 0.01), alpha-2-HS glycoprotein (p value < 0.01), ATP synthase subunit alpha (p value < 0.01), carbonic anhydrase 3 (p value < 0.01), and transgelin (p value < 0.001).

All the differentially expressed proteins that we identified as differentially expressed by shotgun proteomics can be classified according to their main biological function: energy metabolism, myofibrillar proteins, actin microfilament, detoxification and cytoprotection, protein turnover, signal transduction, and serum proteins.

Energy Metabolism—Overall, proteins involved in energy metabolism showed a lower abundance in older women. Indeed, among the 11 proteins showing significant differences, nine were less abundant in older women. Of note, only four of

TABLE II
Significantly differentially expressed proteins of LIS fraction from vastus lateralis muscle of mature and old women, quantified by label-free mass spectrometry

Gene	UniProt accession	Protein name	Ratio (Old/ Mature women)	Quantitation analysis (*p* value)	Western-Blot (*p* value)	Peptides used for quantitation	Mascot Score	Main biological function
ACADM	P11310	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	−1.16	0.01	0.05	2	119	Energy metabolism
ATP5A1	P25705	ATP synthase subunit alpha, mitochondrial	−1.12	0.01	0.05	3	193	Energy metabolism
ATP5B	P06576	ATP synthase subunit beta, mitochondrial	−1.11	0.04		3	107	Energy metabolism
CRAT	P43155	Carnitine O-acetyltransferase	−1.21	0.001		1	50	Energy metabolism
FH	P07954	Fumarate hydratase, mitochondrial	−1.27	0.004		2	163	Energy metabolism
GOT2	P00505	Aspartate aminotransferase, mitochondrial	−1.22	0.02	0.05	9	575	Energy metabolism
LDHB	P07195	L-lactate dehydrogenase B chain	−1.20	0.046		11	831	Energy metabolism
SLC25A4	P12235	ADP/ATP translocase 1	−1.19	0.03		1	38	Energy metabolism
TALDO1	P37837	Transaldolase	−1.16	0.03		1	75	Energy metabolism
FABP4	P15090	Fatty acid-binding protein, adipocyte	1.69	0.002		2	75	Energy metabolism
PFKL	P17858	6-phosphofructokinase, liver type	1.41	0.03		1	58	Energy metabolism
MYH1	P12882	Myosin-1	−1.16	0.002		2	64	Myofibrillar proteins
MYL1	P05976	Myosin light chain 1/3, skeletal muscle isoform	−1.29	0.003		1	50	Myofibrillar proteins
TTN	Q8WZ42	Titin	−1.19	0.01		8	401	Myofibrillar proteins
ANKRD2	Q9GZV1	Ankyrin repeat domain-containing protein 2	1.25	0.02		2	101	Myofibrillar proteins
ACTB	P60709	Actin, cytoplasmic 1	−1.16	0.01		7	342	Actin microfilament
FLNC	Q14315	Filamin-C	−1.15	0.03		3	148	Actin microfilament
ANXA2	P07355	Annexin A2	−1.18	0.03		2	132	Actin microfilament
CFL1	P23528	Cofilin-1	1.47	0.03		1	43	Actin microfilament
TAGLN	Q01995	Transgelin	1.33	0.03	0.001	1	91	Actin microfilament
BLVRB	P30043	Flavin reductase (NADPH)	−1.30	0.01		1	86	Detoxification, Cytoprotection
HSPA4	P34932	Heat shock 70 kDa protein 4	−1.24	0.01		2	61	Detoxification, Cytoprotection
OAS2	P29728	2′−5′-oligoadenylate synthase 2	−1.14	0.04		1	42	Detoxification, Cytoprotection
CA3	P07451	Carbonic anhydrase 3	1.41	0.04	0.05	16	1464	Detoxification, Cytoprotection
APEH	P13798	Acylamino-acid-releasing enzyme	−1.11	0.03		1	41	Protein turnover
RPS27A	P62979	Ubiquitin-40S ribosomal protein S27a	−1.20	0.0002		2	106	Protein turnover
EEF2	P13639	Elongation factor 2	−1.14	0.048		4	159	Protein turnover
PRKAR2A	P13861	cAMP-dependent protein kinase type II-alpha regulatory subunit	−1.18	0.03		1	62	Signal transduction
YWHAE	P62258	14–3-3 protein epsilon	−1.17	0.0003		7	220	Signal transduction
AHSG	P02765	Alpha-2-HS-glycoprotein	−1.23	0.01	0.05	1	55	Serum
FGA	P02671	Fibrinogen alpha chain	−1.20	0.01		4	142	Serum
HV304	P01765	Ig heavy chain V-III region BRO	−1.19	0.046		1	76	Serum
KV402	P01625	Ig kappa chain V-IV region Len	−1.14	0.02		2	128	Serum
C4A	P0C0L4	Complement C4-A	−1.22	0.002		2	115	Serum
HBG1	P69891	Hemoglobin subunit gamma-1	−1.31	0.002		1	69	Serum

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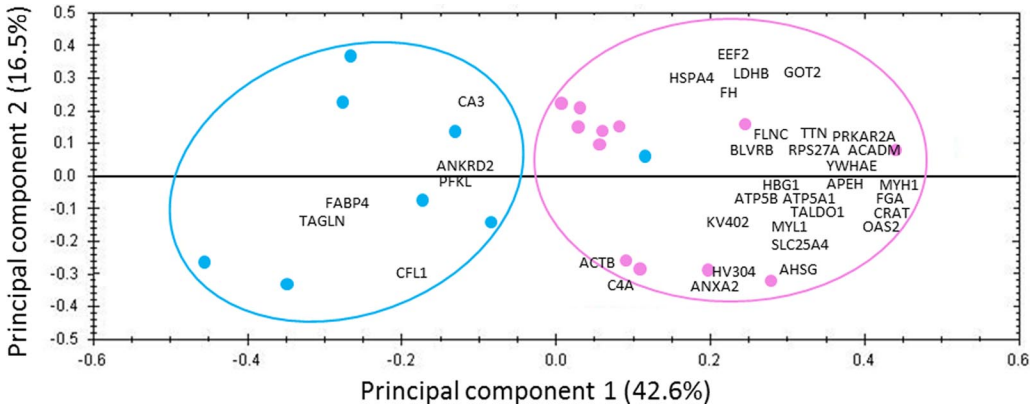

FIG. 3. **Score plot and loadings of principal component analysis from quantified proteins in LIS fraction of *vastus lateralis* from mature and old women.** Discriminations are on principal component 1, and revealed the protein expressions related to muscle aging. The pink line delineates the mature women (pink circles) and the blue line delineates the old women (blue circles). The differentially expressed proteins are marked by abbreviations: ACADM, medium-chain specific acyl-CoA dehydrogenase, mitochondrial; ACTB, actin, cytoplasmic 1; AHSG, alpha-2-HS glycoprotein; ANKRD2, ankyrin repeat domain-containing protein 2; ANXA2, annexin 2; APEH, acylamino-acid-releasing enzyme; ATP5A1, ATP synthase subunit alpha, mitochondrial; ATP5B, ATP synthase subunit beta, mitochondrial; BLVRB, flavin reductase (NADPH); C4A, complement C4-A; CA3, carbonic anhydrase 3; CFL1, cofilin-1; CRAT, carnitine O-acetyltransferase; EEF2, elongation factor 2; FABP4, fatty acid-binding protein, adipocyte; FGA, fibrinogen alpha chain; FH, fumarate hydratase, mitochondrial; FLNC, filamin-C; GOT2, aspartate aminotransferase, mitochondrial; HBG1, hemoglobin subunit gamma-1; HSPA4, heat shock 70 kDa protein 4; HV304, Ig heavy chain V-III region TEI; KV402, Ig kappa chain V-IV region Len; LDHB, L-lactate dehydrogenase B chain; MYH1, myosin-1; MYL1, myosin light chain 1/3, skeletal muscle isoform; OAS2, 2′-5′-oligoadenylate synthase 2; PFKL, 6-phosphofructokinase, liver type; PRKAR2A, cAMP-dependent protein kinase type II-alpha regulatory subunit; RPS27A, ubiquitin-40S ribosomal protein 27A; SLC25A4, ADP/ATP translocase 1; TAGLN, transgelin; TALDO1, transaldolase; TTN, titin; YWHAE, 14–3-3 protein epsilon.

those proteins were previously mentioned in proteomics studies of muscle aging.

L-lactate dehydrogenase B chain catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. The down-regulation during aging of L-lactate dehydrogenase is in agreement with a proteomic study performed on rat gastrocnemius muscle (22), although an up-regulation has also been reported by (23). The down-regulation of L-lactate dehydrogenase is in agreement with reduced lactate dehydrogenase activity previously observed in human muscle during aging (24). Mitochondrial aspartate aminotransferase takes part in amino acid metabolism and in the malate-aspartate shuttle, which is essential for mitochondrial oxidation of glycolytic NADH. In proteomic studies performed on human *vastus lateralis* muscle, mitochondrial aspartate aminotransferase was also found to be down-regulated during aging (9), whereas its cytoplasmic isoform was up-regulated (7). ATP synthase subunits alpha and beta form the catalytic core of mitochondrial membrane ATP synthase, which produces ATP from ADP in the presence of a proton gradient across the membrane. The down-regulation during aging of the ATP synthase chains agrees with previous studies on rat gastrocnemius muscle (22, 25), but disagrees with others that found higher ATP synthase contents in old compared with young muscles (7, 23). In agreement with our observation, Papa (26) reported decreased mitochondrial ATPase activity in old human muscle when compared with young muscle.

All other proteins involved in energy metabolism have not been previously identified in proteomics studies of muscle aging. Transaldolase is implicated in the nonoxidative phase of the pentose phosphate pathway, and is responsible for the generation of NADPH to protect cellular integrity from reactive oxygen species (27). Fumarate hydratase catalyzes the reversible hydration/dehydration of fumarate to malate in the Krebs cycle. ADP/ATP translocase 1 catalyzes the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane. Medium-chain specific acyl-CoA dehydrogenase is involved in mitochondrial fatty acid β-oxidation and is specific for acyl chain lengths of 4 to 16. Carnitine O-acetyltransferase is important for short-chain fatty acid metabolism (28), and has recently been described as a modulator of whole body glucose homeostasis (29). Notably, the muscle-specific loss of carnitine O-acetyltransferase function has been reported to promote insulin-resistance (29), which may be central for the age-related development of metabolic syndrome in the elderly.

Two proteins were more abundant in older women: fatty acid binding protein (FABP), adipocyte; and 6-phosphofructokinase, liver type. The 6-phosphofructokinase protein catalyzes the third step of glycolysis, *i.e.* the phosphorylation of fructose-6-phosphate by ATP to generate fructose 1,6-biphosphate and ADP. Fatty acid binding protein is implicated in intracellular lipid transport (30); FABP adipocyte type was found to be expressed in skeletal muscle but to a lower extend than FABP heart type (31). The up-regulation during

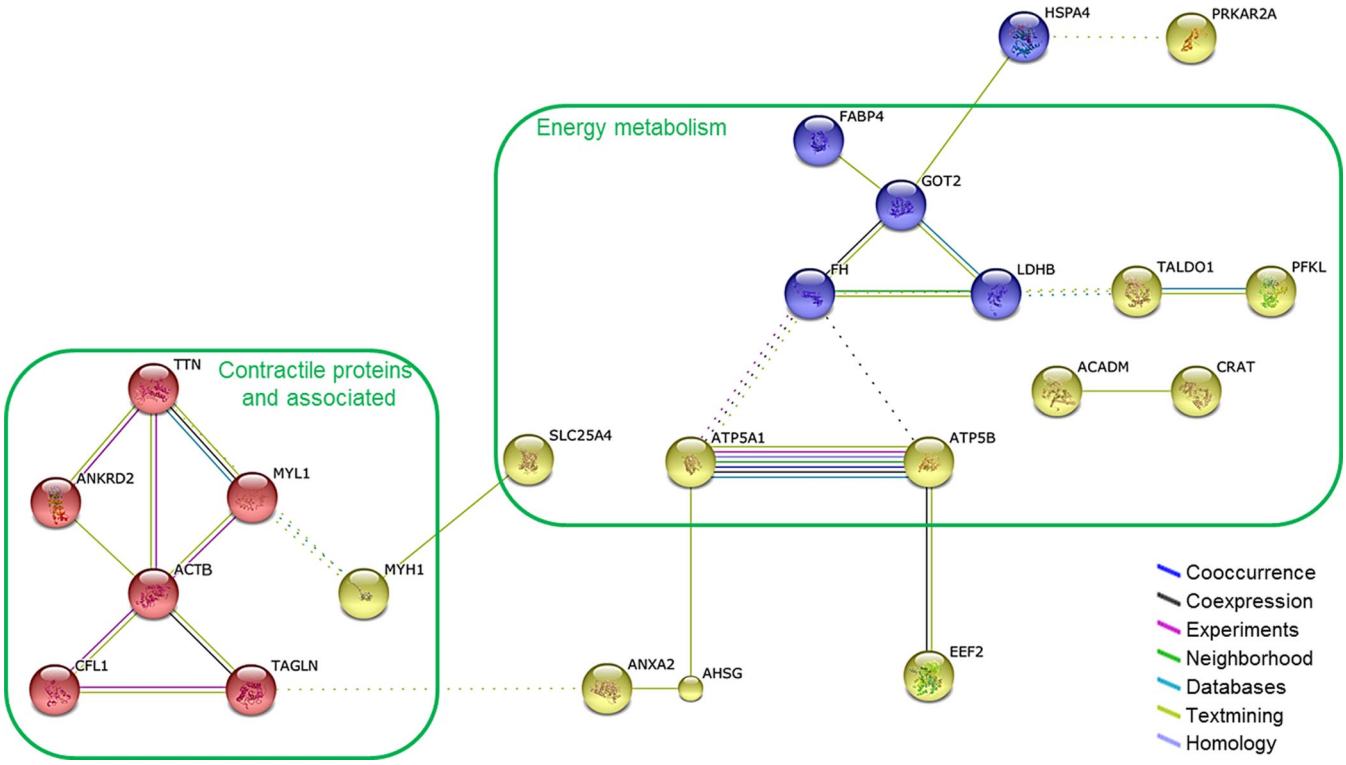

FIG. 4. **Functional interactions network linking the significantly differentially expressed proteins between mature and old women (String database) (20).** K-mean classification revealed three substructures mainly represented by contractile and energy metabolism proteins. The proteins involved in the network are marked by abbreviations: ACADM, medium-chain specific acyl-CoA dehydrogenase, mitochondrial; ACTB, actin, cytoplasmic 1; AHSG, alpha-2-HS glycoprotein; ANKRD2, ankyrin repeat domain-containing protein 2; ANXA2, annexin 2; ATP5A1, ATP synthase subunit alpha, mitochondrial; ATP5B, ATP synthase subunit beta, mitochondrial; CFL1, cofilin-1; CRAT, carnitine O-acetyltransferase; EEF2, elongation factor 2; FABP4, fatty acid-binding protein, adipocyte; FH, fumarate hydratase, mitochondrial; GOT2, aspartate aminotransferase, mitochondrial; HSPA4, heat shock 70 kDa protein 4; LDHB, L-lactate dehydrogenase B chain; MYH1, myosin-1; MYL1, myosin light chain 1/3, skeletal muscle isoform; PFKL, 6-phosphofructokinase, liver type; PRKAR2A, cAMP-dependent protein kinase type II-alpha regulatory subunit; SLC25A4, ADP/ATP translocase 1; TAGLN, transgelin; TALDO1, transaldolase; TTN, titin.

muscle aging of FABP4 may relate to an increased number of adipocytes and/or to an increased expression in skeletal muscle fibers. The over-expression of FABP adipocyte type during muscle aging agrees with previous studies (32, 33) indicating an age-related increase in FABP heart type, and this was interpreted as a shift of old fibers to aerobic oxidative metabolism and slower twitching activity.

Myofibrillar Proteins—Ankyrin repeat domain-containing protein 2 is localized in both the nucleus and the sarcomeric I-band, and is involved in a mechano-signaling pathway that links myofibrillar stress to gene expression (34). It is preferentially expressed in slow type I fibers of human muscle (35), and its up-regulation is in agreement with a fast-to-slow transition during aging. Ankyrin repeat-domain containing protein 2 is also induced by denervation (36). Its up-regulation during muscle aging is then consistent with the development of sarcopenia because an impaired capacity for axonal reinnervation of deinnervated muscle fibers has been shown in aged animals (37).

Although the bulk of the myofibrillar proteins precipitate, a small percentage of them is easily releasable and found in the LIS extract (17). The three myofibrillar proteins, which we identified in muscle LIS extract as differentially expressed during aging, were less abundant in older women: myosin-1; titin, and myosin light chain 1/3, skeletal muscle isoform. Myosin, the motor protein for muscular contraction, is a hexameric protein that consists of two heavy chain subunits (which myosin-1 belongs to), two alkali light chain subunits and two regulatory light chain subunits (which myosin light chain 1/3 belongs to). Titin is essential for myofibrillar assembly by connecting the Z line to the M line in the sarcomere, and represents also a regulatory node for various transduction pathways (38). The function of the easily releasable proteins remains unknown, although they were suggested to represent intermediate products in the turnover of myofibrillar proteins (39, 17). Therefore their reduced levels in old LIS extract may suggest a decreased myofibrillar protein turnover in the old muscle.

Actin Microfilament—Five cytoskeletal proteins were differentially expressed during muscle aging. Actin cytoplasmic 1 (or β-actin), annexin A2 and filamin-C were down-regulated between mature and older women. Only β-actin has been

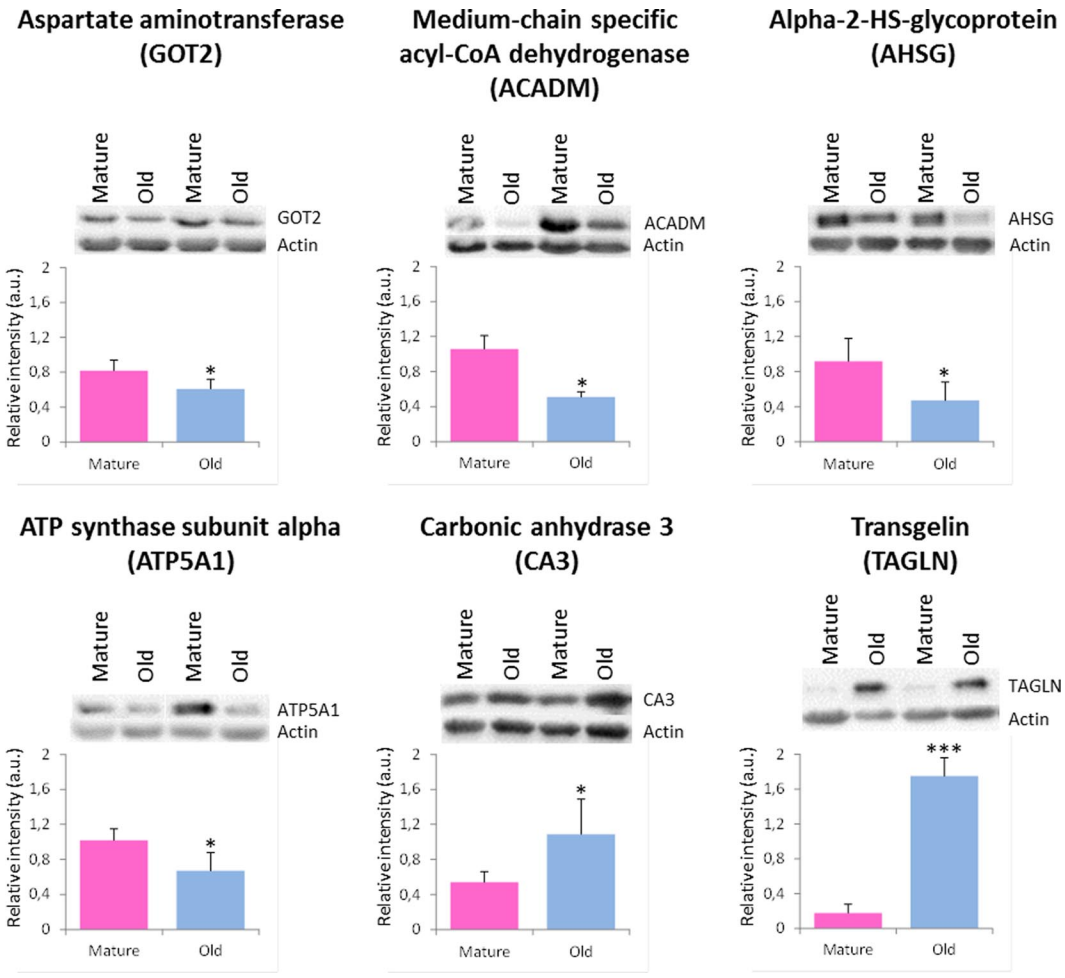

FIG. 5. **Representative Western blot of aspartate aminotransferase (GOT2), medium-chain specific acyl-CoA dehydrogenase (ACADM), alpha-2-HS glycoprotein (AHSG), ATP synthase subunit alpha (ATP5A1), carbonic anhydrase 3 (CA3), and transgelin (TAGLN), between mature (pink) and old (blue) women.** Results are indicated as mean ± standard deviation. Significance: *: p value < 0.05 and ***: p value < 0.001.

previously reported to be modified in a muscle aging study, and was similarly found to decline in old human vastus lateralis muscle (8). Polymerization of β-actin leads to the formation of cytoskeletal actin microfilaments. Annexin A2 can bind specifically to actin and this binding has been linked to the formation and/or stabilization of actin cytoskeleton at cellular membranes (40). Filamin-C is a muscle specific actin-binding protein, that interacts with both Z-disc proteins and sarcolemma-associated proteins and is involved in membrane anchorage of myofibrils. Notably, filamin C mutations are responsible for a myofibrillar myopathy, which preferentially occurs as a result of aging-related impairments in the proteolytic machinery (41).

Two cytoskeletal proteins were more abundant in older women: cofilin-1, and transgelin. Cofilin-1 binds to β-actin and regulates actin cytoskeleton organization (42, 43). Although we observed an age-related increase in the non-muscle isoform, cofilin-1, previous studies in rats demonstrated an increase in the muscle isoform cofilin-2 (44, 45). Transgelin is an actin stress fiber-associated protein that acts to stabilize actin polymers (45). Therefore, our results showed a global age-related deregulation of cytoskeletal proteins that are related to actin microfilaments. Previous studies in rat muscle (4, 46) also showed an altered expression of proteins of the intermediate filament and microtubule network. The differential expression of cytoskeletal proteins during aging may be important for cellular integrity and for the reorganization of contractile structures.

Detoxification and Cytoprotection—Four proteins with detoxification and cytoprotection functions are found to be differentially expressed during aging. Only carbonic anhydrase 3 was more abundant in older women and previously reported in muscle aging studies (44, 8). Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide and this increase with aging is interpreted as a higher demand for efficient CO_2 removal during muscle aging (8). Slow-twitch muscles present higher level of carbonic anhydrase 3, and this observation is in accordance with a fast-to-slow transition of the old skeletal

muscle. The protein 2′-5′-oligoadenylate synthase 2 uses ATP in 2′-specific nucleotidyl transfer reactions to synthesize 2′-5′-oligoadenylates, which activates latent ribonuclease, resulting in degradation of viral RNA and inhibition of virus replication. NADPH-flavin reductase is the fetal form of biliverdine reductase, and has been found to be expressed in adult human skeletal muscle (47, 48). NADPH-flavin reductase catalyzes the NADPH-dependent reduction of FMN, FAD, riboflavin and biliverdin (49), which produces bilirubin, a recognized potent antioxidant (50, 51). Heat shock 70 kDa protein 4 is a chaperone not inducible by heat-shock and a component of the cytosolic protein folding machinery (52). The age-related decrease in NADPH-flavin reductase and heat shock 70 kDa protein 4 may promote ROS-induced oxidative damages and the accumulation of aggregated proteins.

Protein Turnover—Three proteins related to protein turnover were found to significantly decline during aging, elongation factor 2, ubiquitin-40S ribosomal protein S27a, and acylamino-acid-releasing enzyme; none has been previously reported in muscle aging studies. Elongation factor 2 determines the GTP-dependent ribosomal translocation step during translation elongation, and is essential for the regulation of protein synthesis (53). Ubiquitin-40S ribosomal protein S27a is a fusion protein that is post-translationally processed to generate ubiquitin and protein S27a, a component of the 40S ribosome. Ubiquitin-40S ribosomal protein S27a thus contributes not only to the cellular ubiquitin pool and to proteolysis, but also the 40S ribosome and to protein synthesis. In response to ribosomal stress, protein S27a is also important for p53 activation (54). Acylamino-acid-releasing enzyme catalyzes the hydrolysis of the N-terminal peptide bond of an N-acetylated protein to generate a protein with a free N terminus. Acylamino-acid-releasing enzyme acts cooperatively with the proteasome, and its inhibition triggers proteasome down-regulation (55). Acylamino-acid-releasing enzyme is also involved in the degradation of oxidatively damaged proteins (56). The age-related decline in elongation factor 2, ubiquitin-40S ribosomal protein S27a and acylamino-acid-releasing enzyme may then be important for the regulation of protein turnover and oxidative stress during muscle aging (14).

Signal Transduction—Two proteins with signaling functions were less abundant in older women than in mature women: cAMP-dependent protein kinase type II-alpha regulatory subunit, and14–3-3 protein epsilon. cAMP-dependent protein kinase type II-alpha regulatory subunit is involved in cAMP signaling in cells, and has not been previously reported in muscle aging studies. The 14–3-3 protein epsilon belongs to the 14–3-3 family of proteins, which mediate signal transduction by binding to phosphoserine-containing proteins. The 14–3-3 protein epsilon was similarly found to be down-regulated during aging in the gastrocnemius muscle of rats (22).

Serum Proteins—Six serum proteins were found to be less abundant in older women than in mature women: alpha-2-HS-glycoprotein, fibrinogen alpha chain, Ig heavy chain V-III region TEI, and Ig kappa chain V-IV chain Len, complement C4-A protein, and hemoglobin subunit gamma-1. Alpha-2-HS-glycoprotein is involved in endocytosis. Immunoglobulin proteins are involved in the immune response, and complement C4-A protein plays a central role in the activation of the classical pathway of the complement system. Fibrinogen has a double function: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet activation. Hemoglobin subunit gamma-1 was down-regulated between mature and old women; this result agrees with previous studies (57, 33). The decrease in serum proteins during muscle aging is in agreement with an impaired blood-flow distribution in aged fibers (58).

CONCLUSION

The purpose of this study was to elucidate the implication of modifications in the LIS protein fraction in the mechanisms of muscle aging. The label-free analysis revealed that 35 proteins were differentially expressed during aging. Most of these proteins were down-regulated in the muscles of the older women, and were mainly proteins involved in energy metabolism and proteins from the myofilament and cytoskeleton. The functional interactions network linking the differentially expressed proteins between mature and old women provides new insight into the origin of both structural and functional changes induced by aging in skeletal muscle. To our knowledge, this study is the first to apply shotgun analysis to aging in human skeletal muscle. Thanks to this global approach, we identified new proteins linked to sarcopenia, involved in different biological processes such as energy metabolism, muscle process or proteolysis.

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S This article contains supplemental Tables S1 to S3 and Data Files S1 and S2.

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II. Altérations du muscle squelettique chez l'homme âgé sain ou atteint de syndrome métabolique

1. Introduction

Le syndrome métabolique (SM) est un ensemble de facteurs de risque pour les maladies cardiovasculaires et le diabète de type II. Les composants de ce syndrome incluent l'obésité abdominale, la dyslipidémie athérogène, une pression sanguine élevée, une résistance à l'insuline ainsi qu'un état prothrombotique et proinflammatoire (Alberti et al, 2009; Grundy, 2007). Etant donné que le SM augmente de cinq fois le risque de développer un diabète de type II (Stern et al, 2004) et pourrait être à l'origine d'environ 17% des maladies cardiovasculaires (Ford, 2005), une meilleure compréhension des causes et des conséquences de ce syndrome devient un enjeu majeur de santé publique.

Il est connu que le vieillissement est un facteur qui favorise fortement l'apparition du syndrome métabolique. Ce phénomène peut être expliqué par l'altération des différentes fonctions physiologiques observées avec l'âge, et plus particulièrement par les altérations du muscle squelettique. Plusieurs études ont récemment mis en évidence qu'une perte de masse et/ou de force musculaire est fréquemment observée chez les individus âgés atteints de SM (Jurca et al, 2005 ; Moon, 2013; Yang et al, 2012). En plus de la perte de force, une étude a également montré que le syndrome métabolique était associé à une diminution des capacités fonctionnelles musculaires (Vieira et al, 2013). Cependant, les altérations du muscle squelettique chez les personnes âgées atteintes de SM sont peu connues.

L'objectif de ce travail a donc été de mettre en évidence les modifications de la structure des fibres musculaire, du contenu en lipides, de l'activité oxydative et de la vascularisation du muscle squelettique lors du vieillissement musculaire associé ou non au syndrome métabolique. De plus, afin de mieux comprendre les mécanismes moléculaires associés au SM chez les personnes âgées, une étude protéomique comparative a également été réalisée.

2. Résultats principaux

- Contenu en lipides et activité oxydative du muscle squelettique chez les personnes âgées saines ou atteintes de SM (**Publication n°3**)

Chez l'homme, le muscle squelettique est composé de différents types de fibres musculaires : les fibres lentes oxydatives (type I), rapides oxydatives et glycolytiques (type IIA), rapides glycolytiques (type IIX) et les fibres hybrides (type I-IIA et IIA-IIX). Afin de mettre en évidence les altérations du muscle squelettique en fonction des différents types de fibres musculaires, un typage contractile a été réalisé sur environ 400 fibres par individus. Le pourcentage d'accumulation lipidique ainsi que la capacité oxydative d'une enzyme mitochondriale, la cytochrome c oxydase (COX), ont également été déterminés en fonction du type de fibres musculaires. Les résultats montrent que le vieillissement est caractérisé par une atrophie et une déformation des fibres de type II. A l'inverse, une augmentation de l'aire des fibres les plus abondantes (type I et type IIA) ainsi qu'une diminution de l'activité COX dans tous les types de fibres semblent caractériser le syndrome métabolique. De plus, le vieillissement et, plus particulièrement, le SM sont associés à une accumulation élevée de gouttelettes de lipides intramyocellulaires essentiellement dans les fibres de type I. Enfin, les analyses d'imagerie moléculaires nous ont permis de mettre en évidence un changement de la composition lipidique entre nos hommes jeunes, âgés sains et âgés avec SM, alors qu'aucune différence n'a été observée entre les types de fibres musculaires au sein d'un même individu. En conclusion, le vieillissement et le syndrome métabolique affectent de manière différente le muscle squelettique en fonction du type de fibres. L'ensemble de ces modifications peuvent alors contribuer à la perte de masse et de force musculaire liée à l'âge, ainsi qu'à l'augmentation de la prévalence du syndrome métabolique.

- Altérations de la capillarisation du muscle squelettique chez les personnes âgées hypertendues avec ou sans syndrome métabolique (**Publication n°4**)

La capillarisation du muscle squelettique va permettre d'apporter les substances nécessaires au bon fonctionnement musculaire. Une diminution de la densité de ces capillaires a été démontrée dans le muscle squelettique de personnes atteintes d'hypertension. Bien que l'hypertension soit le composant du syndrome métabolique le plus prévalent chez la personne âgée, peu de données sont connues quand à l'impact de cette physiopathologie, associée ou non au syndrome métabolique, sur le muscle squelettique lors du vieillissement. Nos résultats montrent que l'hypertension chez les personnes âgées est associée à une raréfaction des

capillaires, une diminution de la capacité des échanges entre les capillaires et les fibres musculaires ainsi qu'à une diminution de la tortuosité. Une baisse de l'activité COX et une augmentation de l'aire de la matrice extracellulaire caractérisent également les personnes âgées hypertendues. Cependant, en comparant les personnes âgées hypertendues avec ou sans syndrome métabolique, nous n'avons observé aucune différence significative quand au contenu en capillaire, à l'activité COX. Ceci suggère alors que l'hypertension semble être majoritairement responsable des altérations vasculaires ainsi que de la diminution de la capacité oxydative du muscle squelettique associées au syndrome métabolique lors du vieillissement. Ainsi, mieux comprendre les mécanismes moléculaires responsables de l'apparition de l'hypertension chez la personne âgée permettrait de prévenir le développement d'autres pathologies comme le syndrome métabolique, ce qui contribuerait à améliorer la qualité de vie des personnes âgées.

- Etude protéomique du vieillissement musculaire associée ou non au syndrome métabolique (**Publication n°5**)

Cette étude nous a révélé que, parmi un total de 586 spots communs entre tous les individus, 88 sont exprimés différentiellement et 78 ont été identifiés par spectrométrie de masse (nanoLC-MS/MS), ce qui correspond à 43 protéines différentes. Les résultats indiquent que le vieillissement chez l'homme est associé à une diminution de l'expression de plusieurs enzymes glycolytiques (ex, glycéraldéhyde-3-phosphate, β-énolase, triosephosphate isomérase), ainsi qu'à des modifications de l'expression de protéines des myofilaments et du cytosquelette (ex, chaînes lourdes de myosine 6B, troponine T, ANKRD2) semblant traduire une transition vers un phénotype musculaire plus lent. De plus, une surexpression de plusieurs protéines impliquées dans la cytodétoxification/cytoprotection (ex, aldéhyde déshydrogénase, HSPA9/A1A/B6, carbonic anhydrase 3) et dans la réparation membranaire (TRIM72, PTRF) est également observée lors du vieillissement. Chez les personnes âgées, le syndrome métabolique est caractérisé par des perturbations au niveau du métabolisme lipidique (ex, acyl-CoA déshydrogénase, FABP3) et par une augmentation de l'expression de certaines protéines impliquées dans le système ubiquitine protéasome (ex, sous unités alpha type-1 et beta type-4 du protéasome). A notre connaissance, ce travail décrit pour la première fois les changements du protéome musculaire liés au syndrome métabolique chez les personnes âgées et l'ensemble des protéines identifiées représente des biomarqueurs potentiels du

vieillissement et du SM pouvant constituer de nouvelles cibles préventives et/ou thérapeutiques.

Publication 3

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"Skeletal muscle lipid content and oxidative activity in relation to muscle fiber type in aging and metabolic syndrome"

En cours de soumission

Skeletal muscle lipid content and oxidative activity in relation to muscle fiber type in aging and metabolic syndrome

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Running Head: Human muscle aging and metabolic syndrome.

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ABSTRACT

One of the most noticeable effects of aging is the reduction of skeletal muscle mass and strength (sarcopenia). Old subjects also exhibit an increased risk of developing metabolic syndrome (MS), which can lead to cardiovascular diseases and to increased mortality. However despite the implication of skeletal muscle in insulin sensitivity, the effects of MS on skeletal muscle in old individuals have poorly been investigated. Immunohistochemical studies and mass spectrometry imaging were performed with muscle biopsies from young (22 years) and old (73 years) men with and without MS, to reveal the importance of age-dependent and MS-associated modifications on fiber-type characteristics. An atrophy of type-II fibers (-28%, and -32% for type IIA and IIX fibers, respectively) and altered fiber shape characterized muscle aging in lean healthy men. In contrast, increased cross sectional area of the most abundant type-I and type-IIA fibers, and reduced COX activity in all fiber types characterized MS, even in active elderly men. Aging and particularly MS were associated with accumulation of intramyocellular lipid droplets. Although lipids mostly accumulated in type-I fibers, matrix-assisted laser desorption/ionization mass spectrometry imaging of intramyocellular lipids did not distinguish fiber types, but clearly separated young, old and MS subjects. In conclusion, aging and MS differently affect skeletal muscle at a fiber-type specific level. Overall these fiber type-specific modifications may be important both for the age-related loss of muscle mass and strength, and for the increased prevalence of MS in elderly men.

Keywords: Metabolic syndrome; aging; skeletal muscle; fiber types; lipid droplets.

INTRODUCTION

Metabolic syndrome (MS) is a clustering of interrelated risk factors for cardiovascular diseases and type 2 diabetes. MS components include abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, elevated glucose, insulin resistance, and a prothrombotic and proinflammatory state (17, 1). The risk for type 2 diabetes being five times more likely in individuals with the syndrome, a better understanding of the causes and mechanisms of MS is a major public health challenge. Several elements have been implicated in the apparition of MS, including genetic abnormalities, lifestyle, improper nutrition, and also aging. A large survey of the US population in fact pointed out that MS prevalence increases with advancing age (12).

Aging affects most tissues and physiologic functions; however, the gradual loss of skeletal muscle mass and functionality (referred to as sarcopenia) is one of the more consistent hallmarks of aging (26). Notably skeletal muscle, which is the most abundant tissue in the adult body, is a major site of fatty acid oxidation and insulin-mediated glucose disposal. Moreover, dysregulation of intramyocellular lipid metabolism is strongly related to insulin resistance (43). Hence, the age-dependent loss of muscle mass may trigger severe metabolic side effects potentially important for the development of MS and ultimately of type 2 diabetes during aging.

At the cellular level, human skeletal muscles are of mixed fiber-type composition, as they comprise slow-oxidative (type-I), fast-oxidative-glycolytic (type-IIA), fast-glycolytic (type-IIX), together with hybrid fibers (37). Previous studies in adults (18, 31) established that fibers endowed with higher mitochondrial oxidative activity also have an increased capacity for fatty acid metabolism (uptake, oxidation and storage). Aging at a cellular level involves a decline in both fiber number and size, atrophy being greater for type-II fibers. Muscle aging has also been associated with increased intramyocellular lipids (9, 8, 21) and with

modifications (or not) in mitochondrial oxidative capacity (33). However, the fiber-type specific distributions of lipid content and oxidative capacity have not previously been investigated during aging, although this might be central for a clear understanding of the underlying mechanisms. Furthermore, despite the importance of MS for cardiovascular diseases and type 2 diabetes, especially in elderly patients, the consequences of this syndrome on fiber-type distribution, size, morphometry, intramyocellular lipid content and oxidative activity, have not been described.

We therefore hypothesized that MS in the elderly could alter the structure and function of the skeletal muscle at the fiber-type level. The purpose of the present study was thus to investigate the age-dependent and MS-associated changes in fiber-type characteristics of the skeletal muscle in humans, and to underline at the fiber-type level the major alterations that might contribute for the pathophysiology of MS in elderly patients.

MATERIALS AND METHODS

Ethical approval. The present study (ClinicalTrials.gov Identifier: NCT00759304) was approved by the Medical Ethics Committee of the University Hospital of Saint-Etienne (France), and was performed in accordance with the principles of the revised Declaration of Helsinki. All subjects were fully informed regarding study participation, and they provided written informed consent.

Subjects. Our study included 15 healthy young (YO) men, 15 healthy elderly (EL) men, and 9 elderly men diagnosed with metabolic syndrome (EL-MS) for at least 10 years. EL and EL-MS men were selected from the PROgnostic indicator of cardiovascular and cerebrovascular events (PROOF) cohort which is a prospective longitudinal cohort study of 1011 subjects (4). Subjects were recruited in Saint-Etienne, France. Exclusion criteria were prior myocardial infarction or stroke, heart failure, atrial fibrillation, type 2 diabetes, morbid

obesity (BMI > 35kg/m^2), Parkinson's disease, and any other disease limiting life expectancy to less than 5 years. Dependant elderlies or those living in institution were also excluded. YO subjects were healthy volunteers with the same exclusion criteria than the elderlies of the PROOF cohort. All subjects underwent standard medical examination, standard blood analyses (fasting glucose, HDL cholesterol, triglycerides) and performed a maximal stress exercise before their inclusion in the study.

Definition of the metabolic syndrome (MS). MS was defined according to the National Cholesterol Education Program Adult Treatment Panel III (17), and was diagnosed when 3 of 5 components occurred: waist circumference > 102 cm, triglycerides > 1.7 mmol/l, HDL cholesterol <1.03 mmol/l, blood pressure >130/85 mmHg, and/or fasting glucose > 5.6 mmol/l (1).

Daily energy expenditure (DEE), body composition, VO_{2max} and specific strength. PROOF subjects completed a DEE questionnaire confirming that old men (EL and EL-MS) were physically active. DEE was assessed by a self-administered physical activity questionnaire, with specific emphasis on autonomy and perceived exertion, as previously described (14). DEE was calculated from the questionnaire using the equation: DEE (kJ/kg/day) = Σ(IA x DA), where IA is the intensity of the activity (in J/min/kg), and DA the duration of the activity (in min/day).

Body composition was measured using dual X-ray absorptiometry (DEXA, Hologic QDR-2000, Bedford, MA). VO_{2max} was estimated using a cycle ergonometer as previously described (14). Measurements of maximal knee extension isometric strength and specific strength were performed as described (42), using a Cybex II (Ronkonkoma, NY, USA). The knee extensors muscles mechanical response was recorded with a strain gauge (FN3030,0-2000N, FGP Sensor, Les Clayes sous Bois, France) located at the level of the external malleolus. All measurements were taken from the subject's right leg subsequently biopsied,

with the knee and hip flexed at 90 degrees from full extension. The volume of the right leg was estimated according to (22), and specific strength was calculated as the maximal isometric strength divided by the volume of the right leg.

Histochemical preparation. Needle biopsies were taken from the right *vastus lateralis* muscle under local anesthesia in the morning after an overnight fast. Biopsies were mounted with tissue freezing medium, frozen in isopentane cooled on liquid nitrogen and stored at -80°C. Serial cross-sections (10 μm thick) were performed using a cryostat (Microm International, Francheville, France) at -25°C.

Fiber type, cross-sectional area (CSA) and shape. Two serial cross-sections were labeled with monoclonal antibodies against myosin heavy-chain-I (MHC-I) (A4.951 from Enzo Life Sciences, Villeurbanne, France) or MHC-IIa (N2.261, Enzo life Sciences), and co-labeled with anti-laminin-α1 (Sigma, Saint-Quentin-Fallavier, France) to outline the fibers, and resolved with corresponding secondary antibodies conjugated to Alexa-Fluor 488 or 546 (Invitrogen, Cergy-Pontoise, France). Images were captured with a high-resolution cooled digital DP-72 camera coupled to a BX-51 microscope (Olympus, Rungis, France) at a resolution of 0.64 μm/pixel. Five fields, each containing 80 fibers, were analyzed per subject. The contractile type (I, I-IIA, IIA, IIA-IIX or IIX), CSA and perimeter were determined for each fiber, using the image processing software Visilog-6.9 (Noesis, Gif-sur-Yvette, France) as previously described (32). Thresholding of MHC images distinguished either positive, negative or intermediate MHC-labeling. Type-I fibers were positive only for MHC-I, and type-IIA fibers positive only for MHC-IIa. Type-X fibers were negative for both MHC-I and MHC-IIa. Type I-IIA hybrid fibers were intermediate for both MHC-I and MHC-IIa, while type IIA-IIX hybrid fibers were only intermediate for MHC-IIa labeling. A shape factor ($perimeter^2/4\pi$ CSA) was calculated, a value of 1.0 indicating a circle, and > 1.0 an

increasingly elongated ellipse. This shape factor is the reciprocal of fiber circularity previously used (24, 41).

Cytochrome c oxidase (COX). COX activity, an index of oxidative capacity, was determined histochemically as previously described (34). Briefly, serial muscle cross-sections were incubated for 1 h at 37 °C with 0.05% diaminobenzidine (D-5637, Sigma) in 0.05 M phosphate buffer (pH 7.3), and then dehydrated and mounted with Eukitt (CML, Nemours, France) and xylene (Elvetec Services, Pusignan, France). COX and MHC images were matched using the Visilog-6.9 software and COX optical density was quantified for each fiber.

Intramyocellular lipids. Oil red O (ORO, Sigma) stock solution (500 mg/ml ORO in 60% triethylphostate in water (v/v)) was diluted with 0.67 vol. water and filtered before use. Cross-sections were air-dried, incubated in 4% paraformaldehyde (v/v) for 30 min, washed thrice with PBS, labelled with anti-laminin-α1 and incubated with a secondary antibody conjugated to Alexa-Fluor 488. Cross-sections were then incubated with ORO diluted solution for 20 min, and washed thrice with water. Pictures were rapidly captured and saved as gray-scale images. ORO and MHC images were matched and analyzed with the ImageJ application (http://rsb.info.nih.gov/ij/) to determine the number and mean area of lipid droplets (LD), and a lipid accumulation index (LAI, i.e. the percentage fiber area occupied by LD) for each individual and for each fiber.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI). Muscle cross-sections (10 µm) were collected on conductive indium-tin-oxide-coated glass slides (Bruker Daltonics, Bremen, Germany) for MALDI imaging. Sections were desiccated under vacuum at room temperature and the MALDI matrix (7 mg/ml α-cyano-4-hydroxycinnamic acid, 0.2% trifluoroacetic acid in water/acetonitrile (v/v)) was applied using ImagePrep (Bruker). Mass spectrometry was performed on an Autoflex-Speed MALDI time-

of-flight (TOF)/TOF mass spectrometer with a Smartbeam laser using FlexControl-3.4 and FlexImaging-3.0 softwares (Bruker). For lipid imaging, ions were detected in positive reflectron mode at a mass range of m/z 200-1500 and a sampling rate of 0.63 GS/s. Lateral resolution was 25 µm and 300 laser shots were accumulated per pixel. Spectra were normalized and extracted from regions of interest (ROI) corresponding to the muscular typology. Principal component analyses were performed for normalized intensities of m/z peaks using ClinProTools-3.0 (Bruker). Online Lipidomics Structure Database (http://www.lipidmaps.org/) and SimLipid software-v3.40 (Premier Biosoft International, USA) were used for lipid identification.

Statistical analysis. Values are means $\pm$ SE. YO, EL and EL-MS groups were compared using one-factor analysis of variance (ANOVA), followed by post-hoc Fisher's test for pairwise comparisons between groups. Univariate linear regression analysis determined relationships between variables of interest in either lean (YO and EL) or old (EL and EL-MS) populations. Statistical analyses were performed using XLSTAT (Addinsoft, Paris, France) and significance was set at $P < 0.05$.

RESULTS

Subject clinical characteristics. Table 1 provides the characteristics of the subjects involved in the present study. Body weight, body mass index (BMI) and blood pressure were similar in lean YO and EL, but elevated in EL-MS men. VO_{2max} decreased with aging and in response to MS. There was also an age-related decrease in muscle specific strength. Daily energy expenditure was similar between EL and EL-MS groups. Fasting blood glucose and triglyceride concentrations were significantly higher, and HDL-cholesterol lower for EL-MS when compared to EL. DEXA analyses showed that EL-MS had a higher percentage of body fat mass and a lower percentage of appendicular lean mass when compared to EL.

Aging and MS do not change fiber type proportion. Contractile type, CSA and shape were assessed for 400-500 fibers per individual and for all 39 individuals. MHC specific antibodies were used to distinguish contractile types in human muscle (Fig. 1*A*). For all individuals, type-I and type-IIA fibers were the most abundant, whereas type-IIX and hybrid fibers (type I-IIA and type IIA-IIX) represented minor components (Fig. 1*B*). No difference in fiber type distribution was observed between YO, EL and EL-MS muscles. Therefore neither aging, nor MS in the elderly men significantly altered fiber type composition estimated in muscle cross-sections.

Effects of aging and MS on CSA. While the proportion of the different fiber-types remained fairly constant, fiber CSA were notably different between YO, EL and EL-MS muscles. As shown in Fig. 1*C*, aging alone was associated with decreased CSA for all type-II and hybrid fibers. Type-I fibers thus exhibited the largest CSA in the elderly groups, but not in the YO group. Regression analyses performed for lean (YO and EL) men indicated that single fiber CSA correlated positively with VO_{2max} for type I-IIA ($r = 0.62$, $P < 0.002$), IIA ($r = 0.50$, $P < 0.005$), IIA-IIX ($r = 0.47$, $P < 0.02$) and IIX ($r = 0.52$, $P < 0.003$) fibers. The decline in type-IIA CSA also correlated ($r = 0.50$, $P < 0.005$) with muscle specific strength in lean (YO and EL) men.

When compared to YO, no decrease in CSA was observed for EL-MS muscles, and rather this group revealed a significant increase in type-I CSA. Notably, EL-MS displayed larger CSA of type-IIA and IIA-IIX fibers when compared to EL muscles (Fig. 1*C*). In old (EL and EL-MS) men, CSA of type-IIA fibers correlated positively with waist circumference ($r = 0.47$, $P < 0.02$) and with BMI ($r = 0.53$, $P < 0.008$).

Effect of aging and MS on fiber shape. In parallel to CSA modifications, old muscles often exhibited fibers with flattened or crushed shape (Fig. 2*A*). To quantify this observation, a shape factor was calculated. As a result of aging alone, the shape factor (and therefore

deformation) of muscle cells was found to significantly increase for the most abundant fibers (type-I and type-IIA) and for type-IIX fibers. MS in elderlies did not further alter fiber shape (Fig. 2*B*).

Effect of aging and MS on COX activities. In addition to morphological and contractile properties, aging and MS may also affect the metabolic properties of muscle fibers. COX enzymatic activity is classically used to investigate mitochondrial oxidative activity in skeletal muscle (38). In the present study, aging was not associated with any significant change in COX activity in type I, I-IIA and IIA fibers, while COX significantly declined in glycolytic type IIA-IIX or IIX fibers (Fig. 3*B*). In contrast, the EL-MS group exhibited a strong decrease in COX activity for type I, I-IIA and IIA fibers when compared to the YO and EL groups. In YO individuals, type-I fibers had the highest COX activity, type-IIA were intermediate, and type-IIX had the lowest activity. This pattern tended to be maintained for EL, but not for EL-MS individuals. Indeed, in this group, COX activity was not significantly different between all fiber types.

Changes in intramyocellular lipid content. ORO staining of neutral lipids was carried out to investigate LD (Fig. 4*A*) and to calculate LAI for each subject. Independently of fiber types, LAI was the highest for the EL-MS group, and also tended to increase for EL when compared to the YO group (Fig. 4*B*). At the fiber-type level (Fig. 4*C*), there was a decreasing gradient of lipids from oxidative type-I to glycolytic type-IIX fibers in YO individuals. This pattern tended to be maintained for EL, but not for EL-MS. Indeed in the EL-MS group, LAI was not significantly different between type-IIA and type-IIX fibers. Moreover when compared to EL and YO, EL-MS muscles accumulated even more lipids in Type-I fibers, and also in glycolytic type-IIX and IIA-IIX fibers (Fig. 4*C*).

Modifications in lipid content in each muscle fiber may be due to changes in LD number and/or area. Image analysis did not reveal major modifications in the mean area of

LD (data not shown), but rather an increased number of LD in oxidative type-I fibers of EL when compared to YO muscle (Fig. 4*D*). MS was also associated with an increased LAI and LD number in type-I fibers, and interestingly also in glycolytic IIX and IIA-IIX fibers (Fig. 4*D*). Regression analyses performed for old (EL and EL-MS) men indicated that BMI was positively correlated with LAI ($r = 0.64$, $P < 0.008$) and with LD number ($r = 0.54$, $P < 0.04$) in type-I fibers. LAI also negatively correlated with VO_{2max} for type-I fibers in old men ($r = 0.50$, $P < 0.05$).

Changes in intramyocellular lipid composition. Because fatty-acid composition of skeletal muscle is important for insulin sensitivity, we wondered whether aging and/or MS may alter the global lipid composition of muscles. Moreover, given the differences observed in lipid content and oxidative capacity, we wondered whether lipid composition might differ between fiber types. To test these hypotheses, MALDI-MSI was carried out on muscle cross-sections from YO, EL and EL-MS subjects (Fig. 5). MSI based on sections coated with α-cyano-4-hydroxycinnamic acid matrix allows an optimal resolution of 25 µm, which is not sufficient to work at the fiber scale. Several regions of interest (ROI), containing either type-I or all type-II fibers, were then identified for each biopsy (Fig. 5*A*). Within each sample and for each ROI (I or II), ionic maps of lipids were acquired. Principal component analyses of m/z peaks of ROIs were then performed to assess the homogeneity/heterogeneity of the lipid composition between individuals and fiber types. Principal component analyses of type-I *vs*. type-II ROIs for each individual revealed no significant separation (Fig. 5*B*), and therefore that lipid composition was apparently similar between fiber types. However, principal component analyses revealed clear differences between YO, EL and EL-MS subjects (Fig. 5*B*). The loadings plot (Fig. 5*C*) showed that YO was associated with a co-localization of m/z 226.37 and 416.63, EL with m/z 295.46 and 616.08, and EL-MS with m/z 250.27 and 266.24. Based on Lipidomics and SimLipid databases, most molecules could not be identified with a

mass tolerance < 200 ppm, and only m/z 266.24 could be assigned as either lauroyl-ethanolamine or C14-sphingosine. Therefore, despite no definitive identification, molecular imaging of lipids highlighted several m/z values, which differed between YO, EL and EL-MS muscles. This suggests that aging and MS are associated with notably altered lipid profiles in human skeletal muscle.

DISCUSSION

Aging of skeletal muscles involves both a decrease in fiber size (atrophy) and number (hypoplasia). A preferential loss of fast motor units (containing type-IIA and IIX fibers) prevails after the sixth decade of age (6). Such neuropathic process, leading to type-II denervation and parallel reinervation by type-I motoneuron collaterals, may lead to a new balance between fiber types. Nonetheless, no consensus has been reached in the literature on whether there is a shift towards higher (11, 28, 41), lower (36, 13) or unaltered percentages (30, 25) of type-I *vs*. type-II fibers in human aging. In the present study, immunohistological analyses of MHC expression were used to distinguish the five major types (I, I-IIA, IIA, IIA-IIX, and IIX) of fibers characterizing human skeletal muscle (37), and our data revealed no significant variation in fiber type composition with advancing age. Therefore, the fiber type proportion did not seem a central determinant for the alterations observed in the aged muscle cross-sections. Studies based on isolated single fibers and SDS-PAGE suggested that hybrid fibers co-expressing two MHC isoforms were (10) or were not (27) more frequent in 70-75 year old, or even become prominent in very old (85-98 year old) muscles (3). Using longitudinal cross-section, Andersen (2) in fact indicated that fiber type may switch along the length of the fiber. Such switch could not be detected in transversal cross-sections and our study may therefore underestimate hybrid fibers.

In contrast to fiber type distribution, there is consensus as to the age-dependent variations in fiber area. Previous analyses of human muscle biopsies (11, 41, 36, 7), together with autopsy studies (29), showed that type-II fiber CSA diminishes with age. Considering the subtypes of type-II fibers, a similar atrophy was found for type-IIA and type-IIX fibers (28, 23). Herein we extend this notion and provide evidence that the age-dependent decrease in size similarly occurs for hybrid (type I-IIA and type IIA-IIX) fibers. Image analysis was further used in the present study to demonstrate an altered shape for the main fiber types, a notion previously mentioned but not quantified in the old muscle (2). The reasons for such an altered shape or circularity are unknown, but an altered shape may represent the first signs of fiber disuse, cell death or reorganization of motor units in the old skeletal muscle (24, 2).

In addition to changes in contractile proteins, mitochondrial dysfunction and lipid accumulation have been implicated in the age-related decline of muscle functionality (33). Mitochondria are the main producers of cellular energy. Declines in mitochondrial oxidative capacity were reported with advancing age (38), although physical activity, rather than chronological age, was also reported to be the primary determinant (33). Age-dependent modifications in oxidative activity could also affect the fiber types differently, but this point was not previously investigated. We measured COX activity, an inner mitochondrial membrane-embedded enzyme, to estimate oxidative metabolism, and could not detect any age-related change in slow oxidative fibers of healthy lean subjects. We did find reduction in COX activity, but surprisingly this was limited to fast-glycolytic fibers. Because of low proportions of IIA-IIX and IIX fibers in human muscle, this fast-specific reduction in COX capacity may not dramatically affect the global COX activity in skeletal muscle.

Besides subcutaneous fat, muscles contain lipids located between and within muscle fibers. Previous investigations indicated that intermuscular fat increases with advancing age (21), and magnetic resonance spectroscopy (9) and ultrastructural (8) studies showed that

elderlies have greater intramyocellular lipid. However fat distribution among fiber-types was not previously assessed in the old muscle. Our data indicate that intramyocellular lipids follow a similar pattern than oxidative activity in relation to fiber type in lean old men, and this is likely related to the increased capacity of type-I fibers for fatty acid metabolism. Our study also reveals that, despite similar BMI, blood pressure and fiber oxidative activity, healthy old men tend to increase total LAI, when compared to young men. As previously shown by ultrastructural investigations (8), one possibility could be that a lower proportion of mitochondria in contact with lipid droplets partly account for such altered lipid metabolism. Overall, these data suggest that healthy aging is mostly associated with type II fiber atrophy and altered fiber shape. These characteristics notably differed with MS in elderly men.

MS is prevalent in elderly people (12), and has substantial implications for health care, as it increases the risk for type 2 diabetes, cardiovascular diseases (20), and mortality from all causes (40). In elderly men, MS is also associated with muscle weakness (44), but the consequence of this pathology on muscle fiber characteristics has not been investigated before. In fact, most studies relating fiber characteristics to obesity and/or type 2 diabetes have been performed with adults (18, 31), and none with elderly subjects. Our study now reveals that MS in elderly men, who are physically active, is sufficient to strongly alter the characteristics of skeletal muscle at the fiber-type level.

A major difference between aging and MS reported here is related to fiber CSA. CSA of oxidative-glycolytic (type-IIA and IIA-IIX) fibers increase with MS in old patients, but decrease with aging. Although there is no previous report for MS, a similar larger CSA was reported for type-II fibers in obese adults (31, 15). Another major difference that we observed between MS and aging is related to COX activity, which is notably affected in oxidative fibers of EL-MS men. In obese adults, a reduced proportion of oxidative fibers (39), or alternatively a lower oxidative activity (18), were suggested as determinant for global muscle

oxidative capacity. In elderly men, our data suggest that this is not fiber distribution but rather low oxidative activity within each fiber type which characterizes MS. If EL-MS muscles do not preserve oxidative metabolism and microcirculation, an increased CSA could have deleterious effects on the oxygen and substrate supply to the center of the EL-MS fiber, leading to reduced functionality such as specific strength.

Because mitochondria play a central role in fatty acid oxidation, a reduced oxidative enzyme capacity is typically associated with a reduced capacity for lipid oxidation and an accumulation of intramyocellular lipids (18, 39), which is consistent with our results in EL-MS men. Although lipids essentially accumulate in oxidative type-I fibers, our data also revealed increased lipids in glycolytic fibers. The significance of such lipid accumulation in type-IIX fibers is not understood yet, although this was previously observed in obese adults (18, 31). In addition to intramyocellular lipid accumulation, lipid composition is also central for insulin resistance. Previous studies indeed reported that both insulin-resistant obese patients and insulin-sensitive athletes exhibit increased intramyocellular lipids, but the formers accumulate saturated and the latters polyunsaturated fatty acids in muscle phospholipids (16, 5, 19). In addition other aspects, such as lipid peroxidation, undoubtedly play an important role in insulin resistance (35). These observations are in accordance with our data that both aging and MS alter muscle lipid composition, as revealed by molecular imaging. In contrast, we could not detect major differences in lipid composition between fiber types, despite fiber-specific divergences in oxidative metabolism and lipid content. The diffusion of lipids in vivo between neighboring fibers could partly account for such homogeneity in lipid composition.

In summary, atrophy of type-II fibers and altered fiber shape essentially characterize muscle aging in lean healthy men. In contrast, increased CSA of the most abundant type-I and IIA fibers, and reduced COX activity in all fiber types characterize MS, even in active elderly

men. Aging and particularly MS are further associated with an accumulation of intramyocellular lipids and with altered lipid compositions. Therefore, our study suggests that MS in the elderlies significantly alters the structure and function of the skeletal muscle at the fiber-type level. Overall these fiber type-specific modifications may be important both for the age-related loss of muscle mass and strength, and for the increased prevalence of MS which may ultimately lead to insulin resistant and type 2 diabetes in elderly subjects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.G., C.C.G. and D.B. contributed to the conception and design of the research; J.V., L.F., F.R. and J.C.B. performed clinical research. M.G., C.C.G. and L.T. performed the experiments; M.G., C.C.G. and D.B. analyzed the data; M.G., C.C.G. and D.B. interpreted the results of the experiments; M.G. and L.T. prepared the figures; M.G. and D.B. drafted the manuscript; M.G., C.C.G., L.T., B.M., C.B., L.B., D.T., C.P., D.A., B.P., J.V., F.R., L.F., J.C.B. and D.B. edited, revised and approved the final version of the manuscript.

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FIGURE LEGENDS

Fig. 1. Effects of aging and metabolic syndrome on fiber type distribution and cross-section area. Vastus lateralis biopsies were from young (YO), elderly (EL) and elderly men with metabolic syndrome (EL-MS). ***A***: Representative images of serial cross-sections labeled (red) for either myosin heavy-chain I (MHC-I, upper) or MHC-IIa (lower). Cross-sections were counter-stained for laminin-α1 (green) to outline the fibers. Thresholding of MHC images distinguished either positive, negative or hybrid MHC-labeling. Type-I fibers (¶) were positive only for MHC-I, and type-IIA fibers (^) positive only for MHC-IIa. Type-IIX fibers (#) were negative for both MHC-I and MHC-IIa. Fiber type proportion (***B***) and cross-section area (***C***) were measured for 400 fibers per subject. Results are means ± SE (n = 9-15). Different letters indicate significant difference ($P < 0.05$) between fiber types. *$P < 0.05$ *vs*. YO group. $^{\S}P < 0.05$ *vs*. EL group.

Fig. 2. Aging alters fiber shape in human muscle. ***A***: Representative images of flattened fibers (†) observed for type-I (upper panel) and type-IIA (lower panel) fibers, in elderly (EL) and elderly men with metabolic syndrome (EL-MS), but not in young (YO) men. ***B***: Shape factor (perimeter^2/4π x cross-section area) was measured for 400 fibers per individual in YO, EL and EL-MS muscles. Results are means ± SE (n = 9-15), and different letters indicate significant difference ($P < 0.05$) between fiber types. *$P < 0.05$ *vs*. YO group.

Fig. 3. Effects of aging and metabolic syndrome on cytochrome c oxidase (COX) activity in different fiber types. ***A***: Representative images of serial cross-sections labeled for myosin heavy-chain I (MHC-I, upper), MHC-IIa (middle) and COX activity (lower). MHC labeling (red) was counter-stained for laminin-α1 (green) and used to determine fiber type in young

(YO), elderly (EL) and elderly men with metabolic syndrome (EL-MS). Type-I (¶), type-IIA (^) and type-IIX (#) fibers are indicated. ***B***: COX activity was measured for 400 fibers per individual. Results are means ± SE (*n*=9-15). Different letters indicate significant difference ($P < 0.05$) between fiber types. *$P < 0.05$ *vs*. YO group. $^{\S}P < 0.05$ *vs*. EL group.

Fig. 4. Effects of aging and metabolic syndrome on intramyocellular lipids. ***A***: Representative images of serial cross-sections labeled for myosin heavy-chain I (MHC-I, upper), MHC-IIa (middle), or stained with Oil red O (ORO, lower) for neutral lipids. MHC labeling (red) was counter-stained for laminin-α1 (green) and used to determine fiber type in young (YO), elderly (EL) and elderly men with metabolic syndrome (EL-MS). ORO-stained lipid droplets were viewed as distinct spots and converted to grayscale for quantification. Type-I (¶), type-IIA (^) and type-IIX (#) fibers are indicated. Lipid accumulation indexes (LAI), expressed as percentage of fiber area occupied by lipid droplets, were calculated for whole cross-sections (***B***), and at the fiber type level (***C***). ***D***: The number of lipid droplets per fiber was determined for each fiber type. Results are means ± SE (*n*=9-15). Different letters indicate significant difference ($P < 0.05$) between fiber types. *$P < 0.05$ *vs*. YO group. $^{\S}P < 0.05$ *vs*. EL group.

Fig. 5. Effects of aging and metabolic syndrome on lipid composition in different fiber types. ***A***: Representative images of vastus lateralis cross-sections from young (YO), elderly (EL) and elderly with metabolic syndrome (EL-MS). Regions of interest I (pink) indicate fibers immuno-labeled for MHC-I, while regions of interest II (yellow) indicate fibers immuno-labeled for MHC-IIa. ***B***: Lipid spectra were acquired and principal component (PC) analysis of normalized peak intensities were generated for 100 spectra for regions of interest I and II of YO, EL and EL-MS subjects. There was no separation of fiber types, but a good separation between YO, EL and EL-MS according to PC1 and PC4 (30.6% and 5.5% of explained

variance, respectively). Loadings plot (***C***) and ion density maps (***D***) of the molecules implicated in this separation.

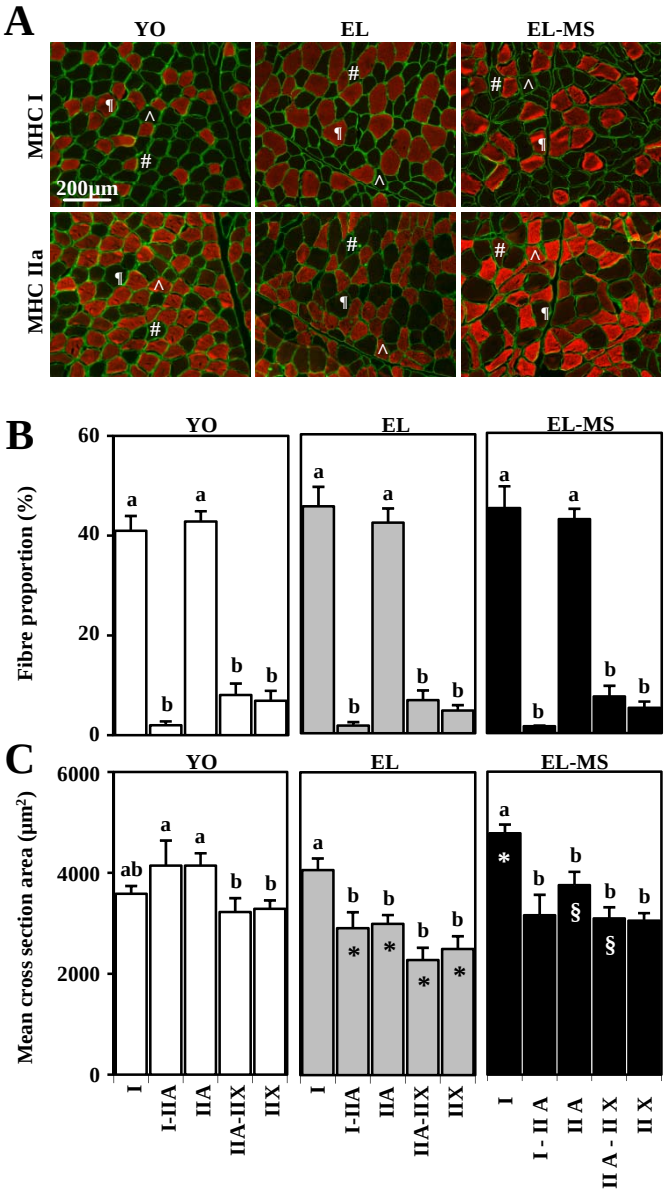
A
YO
EL
EL-MS
MHC I
MHC IIa
200µm
B
Fibre proportion (%)
C
Mean cross section area (µm²)
I
I-IIA
IIA
IIA-IIX
IIX
I - II A
II A
II A - II X
II X

Figure 1

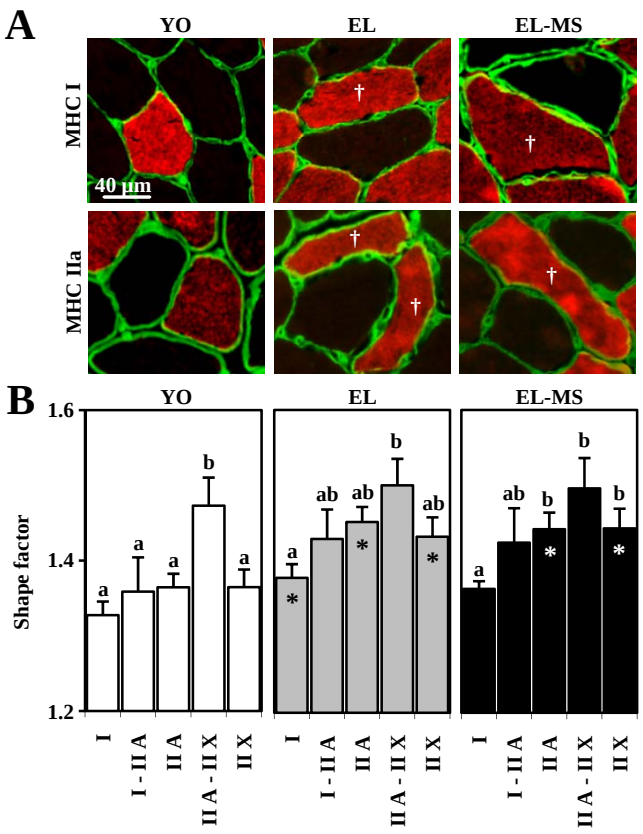
A
YO
EL
EL-MS
MHC I
MHC IIa
40 µm
†
B
YO
EL
EL-MS
Shape factor
1.6
1.4
1.2
a
ab
b
*
I
I - II A
II A
II A - II X
II X

Figure 2

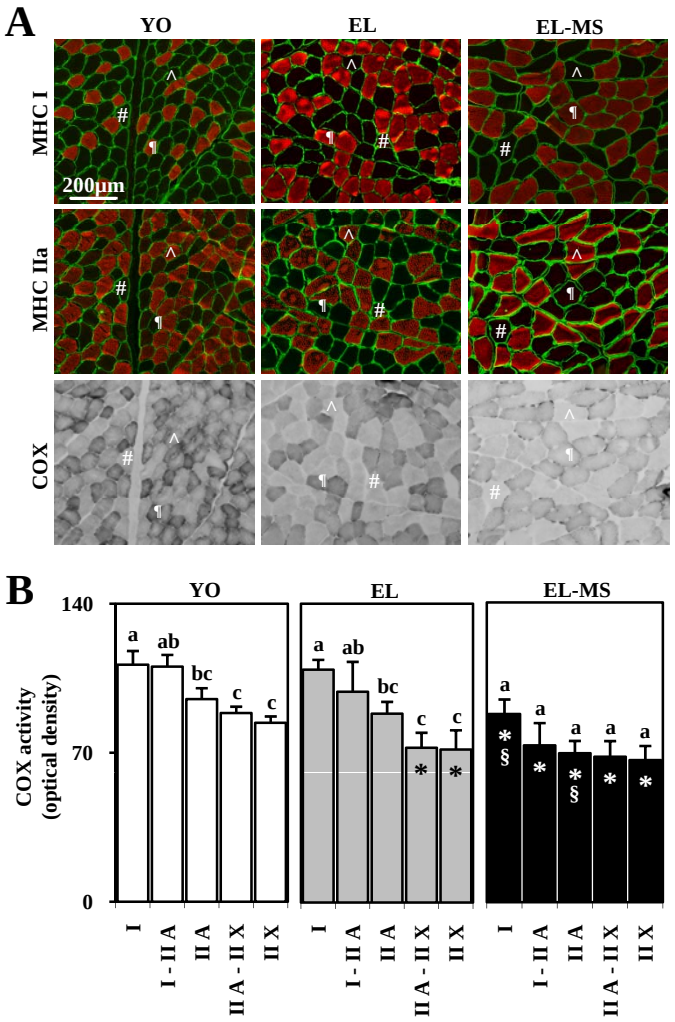
A
YO
EL
EL-MS
MHC I
MHC IIa
COX
200µm
B
COX activity (optical density)
140
70
0
a
ab
bc
c
I
I - II A
II A
II A - II X
II X

Figure 3

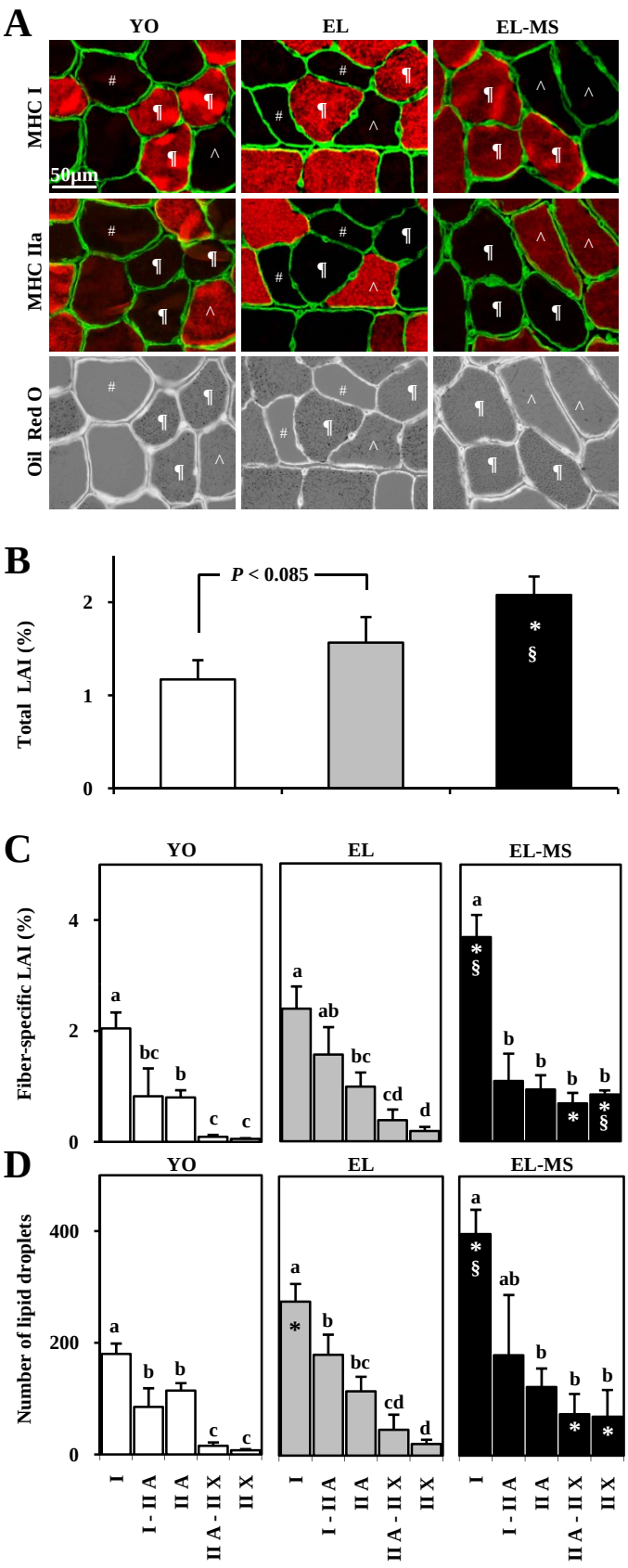
A
YO
EL
EL-MS
MHC I
MHC IIa
Oil Red O
50µm
B
P < 0.085
Total LAI (%)
C
Fiber-specific LAI (%)
D
Number of lipid droplets
I
I - II A
II A
II A - II X
II X

Figure 4

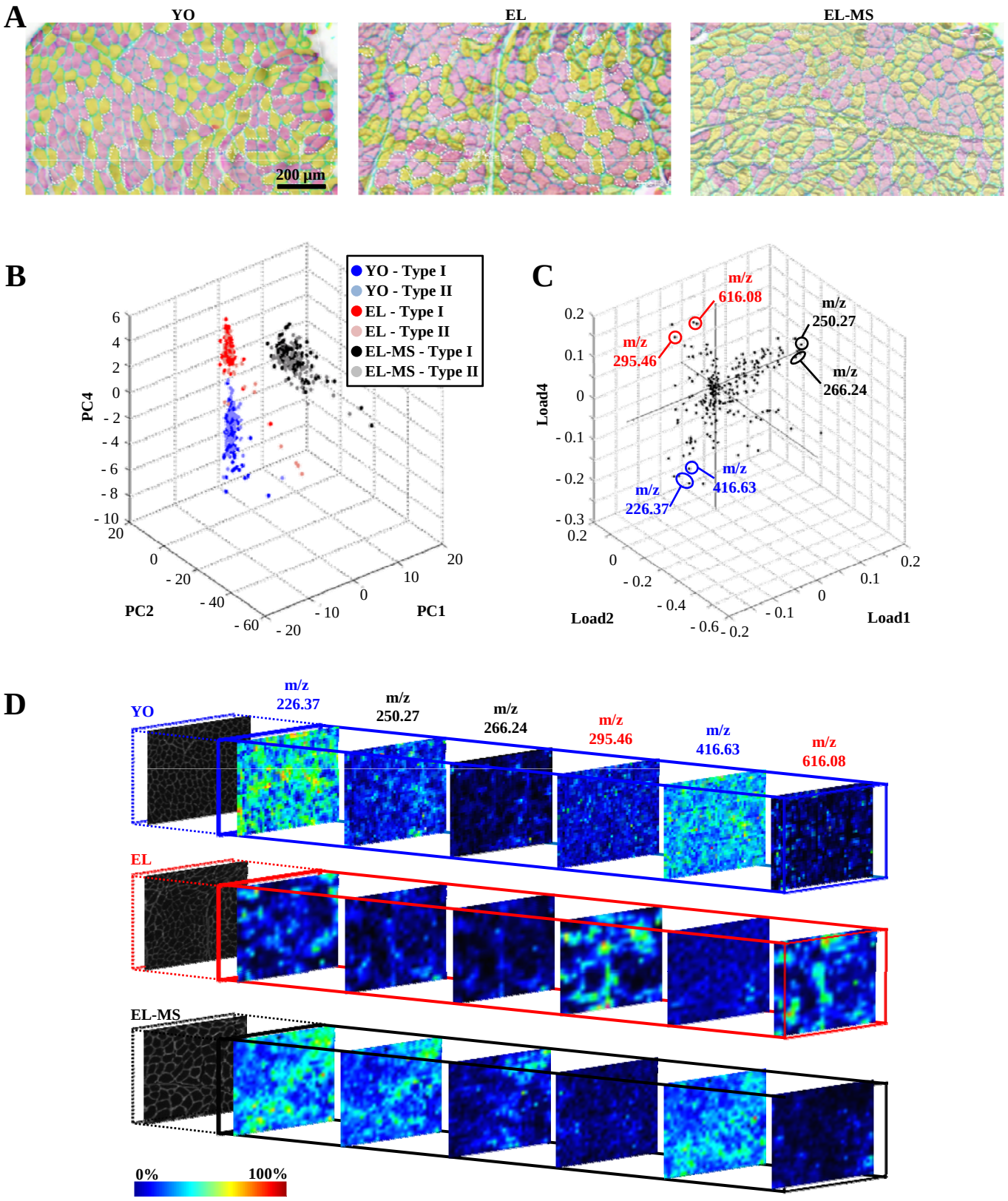
A
YO
EL
EL-MS
200 µm
B
YO - Type I
YO - Type II
EL - Type I
EL - Type II
EL-MS - Type I
EL-MS - Type II
PC4
PC2
PC1
C
m/z 616.08
m/z 250.27
m/z 295.46
m/z 266.24
m/z 416.63
m/z 226.37
Load4
Load2
Load1
D
YO
EL
EL-MS
m/z 226.37
m/z 250.27
m/z 266.24
m/z 295.46
m/z 416.63
m/z 616.08
0%
100%

Figure 5

Publication 4

Marine Gueugneau, Cécile Coudy-Gandilhon, Bruno Meunier, Lydie Combaret, Daniel Taillandier, Cécile Polge, Didier Attaix, Brigitte Picard, Julien Verney, Frédéric Roche, Léonard Féasson, Daniel Béchet.

“Lower skeletal muscle capillarization in hypertensive elderly with or without metabolic syndrome”

En cours de préparation

Lower skeletal muscle capillarization in hypertensive elderly with or without metabolic syndrome

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Running title: Lower muscle capillarization in hypertensive elderly

Key words: hypertension, aging, metabolic syndrome, capillarization, skeletal muscle fiber.

Summary

Aging strongly affects the skeletal muscle and is associated with microvascular dysfunctions. Age is also a primary risk factor for metabolic syndrome (MS), which is a cluster of metabolic and cardiovascular symptoms. Among all MS components, hypertension is the most prevalent in elderly subjects and has a central role in vascular alterations. Despite critical clinical outcomes, the effects of hypertension and MS on skeletal muscle capillarization have poorly been investigated. In the present study, human muscle biopsies from healthy young and elderly men, and elderly men with hypertension or metabolic syndrome were assessed for capillary density (CD), capillary to fiber ratio (C/F), number of capillaries around each fiber-type (CAF), capillary-to-fiber perimeter exchange (CFPE), length of capillary contact to perimeter of fiber (LC/PF), and capillary tortuosity (CapTor). As ECM can modulate transcapillary exchanges and thereby muscle mitochondrial oxidative capacity, we also investigated extracellular matrix (ECM) area and cytochrome c oxidase activity (COX). Our findings reveal that hypertension in elderlies is associated with capillary rarefaction and with decreased capillary-to-fiber interface and tortuosity in skeletal muscle. Lower COX activity and larger ECM area also characterized hypertensive old men. However, no major modification was further observed when hypertension was compared to metabolic syndrome in elderly men. Collectively, our results suggest that hypertension plays a central role in perturbations of skeletal muscle capillarization in aging, and that the other components of metabolic syndrome do not make major additional changes in the aged skeletal muscle capillary network.

Introduction

Hypertension has a central role in vascular alterations. Indeed, hypertension is characterized by structural and functional changes in the microvasculature (Feihl *et al.* 2006; Jonk *et al.* 2007; Serne *et al.* 2007) which includes arterioles, capillaries and venules. In hypertensive adults, the mechanisms regulating vasomotor tone are abnormal, leading to enhanced vasoconstriction, reduced vasodilatation of arterioles (Panza *et al.* 1995; Cardillo *et al.* 2002; Hansen *et al.* 2011), and microvascular remodeling (Hernandez *et al.* 1999; Feihl *et al.* 2008). In addition, a reduction in the number of arterioles or capillaries within vascular beds, called vascular rarefaction, can be observed in hypertensive humans (Henrich *et al.* 1988; Hedman *et al.* 2000; Feihl *et al.* 2006; Hansen *et al.* 2010). In skeletal muscle, capillarization is representative of the potential for exchange of respiratory gases, fuel and metabolites, so it is an important determinant of maximal oxygen consumption, insulin sensitivity, and thus of muscle functionality.

Skeletal muscle, which is the most abundant tissue in the body, is one of the most affected organs during the aging process. Concomitant with the loss of muscle mass and function, aging is often associated with microvascular dysfunctions (Proctor *et al.* 1995; Bearden 2006), which likely contribute to a decline in the physical capacities in elderly subjects. Few reports, investigating fiber type-specific muscle capillarization in humans, have shown that the number of capillaries around a single fiber (CAF) decreased with aging for type II, but not for type I fibers (Coggan *et al.* 1992a; Croley *et al.* 2005; Ryan *et al.* 2006). Results about capillary density (CD) are more conflicting, as both lower CD (Coggan *et al.* 1992a; Frontera *et al.* 2000) and similar CD (Proctor *et al.* 1995; Croley *et al.* 2005; Ryan *et al.* 2006) were reported for human muscle with aging.

Age is also a primary risk factor for metabolic syndrome (MS), which is a cluster of metabolic and cardiovascular symptoms that increase the risk for type 2 diabetes, cardiovascular diseases, and mortality from all causes. MS components include abdominal obesity, atherogenic dyslipidemia, elevated glucose, a prothrombotic and proinflammatory state, and an elevated blood pressure. Among all components of MS, hypertension is the most prevalent in elderly subjects (Ford *et al.* 2010). Despite critical clinical outcomes, the effects of hypertension on skeletal muscle capillarization in old individuals and, the impact of hypertension in the muscle capillary network of elderly with MS have poorly been investigated, and we hypothesized that these vascular dysfunctions in old men with MS are mainly due to hypertension.

The aim of the present study was thus to highlight alterations of the capillary network in skeletal muscle of lean healthy elderly and old men with hypertension or MS. Collectively, our results indicate that hypertension in elderly is associated with lower muscle capillarization in a fiber type-specific manner, which likely contributes to the decline in muscle functionality during aging, but no additional effect of the other components of metabolic syndrome was observed.

Results

Subject clinical characteristics.

Characteristics of the subjects involved in the present study are provided in Table 1. Body weight and body mass index (BMI) were similar between YO, EL and EL-HT, but elevated for EL-MS men. VO_{2max} decreased with aging and in response to MS, but was similar between the EL and EL-HT groups. Blood pressure was similar between healthy YO and EL men, but was elevated for EL-HT and EL-MS men. Muscle specific strength decreased only with aging, and was similar between EL, EL-HT and EL-MS men. Similarly, daily energy expenditure was similar between EL, EL-HT and EL-MS groups. Waist circumference and fasting blood glucose concentrations were significantly higher, and HDL-cholesterol lower for EL-MS when compared to elderly with or without hypertension. DEXA analyses showed that hypertension was not associated with significant modification in body fat mass or appendicular lean mass. However, EL-MS had a higher percentage of body fat mass and a lower percentage of appendicular lean mass when compared to EL and EL-HT subjects.

Muscle fibers characteristics.

Contractile type and CSA were assessed for 400-500 fibers per individual (Table 2). For all individuals, type I and IIA fibers were the most abundant, whereas type IIX and hybrid fibers (type I-IIA and IIA-IIX) represented minor components in the vastus lateralis muscle. No difference in fiber type distribution was observed between YO, EL, EL-HT and EL-MS muscles.

As shown in Table 2, aging alone was associated with decreased CSA for all type II and hybrid fibers. Hypertension alone in elderly subjects was not associated with major modification in fiber-type CSA. However, EL-MS displayed larger CSA for type IIA and IIA-IIX fibers when compared to EL muscles. EL-MS also revealed a significant increase in CSA of type I fibers when compared to YO muscles.

Capillary density and capillary to fiber ratios.

Global indices of capillarization, represented by capillary density (CD) and capillary to fiber ratio (C/F), provide a fiber-type independent estimation of muscle capillary supply. As shown in Fig. 1, CD was not significantly different between YO, EL and EL-HT groups, but was lower for EL-MS compared to EL subjects (Fig. 1A). Global C/F was similar between YO and EL subjects, but this index was significantly lower for EL-HT and EL-MS when compared to healthy EL subjects (Fig. 1B).

Alike at the fiber-type level, the number of capillaries around each fiber-type (CAF) was similar between healthy YO and EL subjects, while in elderly subjects, hypertension was associated with a 24, 26 and 43 % decrease in CAF for type I, IIA and IIX fibers, respectively (Fig. 2B, EL-HT vs. EL). In elderly subjects, metabolic syndrome did not further change CAF for type I, IIA and IIX fibers, as no significant difference was observed between EL-HT and EL-MS fibers.

Regression analyses performed for old men (EL, EL-HT and EL-MS) indicated that VO_{2max} correlated positively with CD ($r = 0.86$, $P < 0.001$) and C/F ($r = 0.79$, $P < 0.001$) and with CAF for type I ($r = 0.5$, $P < 0.026$) and type IIA fibers ($r = 0.6$, $P < 0.005$).

Overall these results suggested that healthy aging is not associated with major alteration in capillary density. They also revealed that, in elderly subjects, hypertension and metabolic syndrome are related to capillary rarefaction in the skeletal muscle.

Capillary-to-fiber interface and capillary tortuosity.

The capillary-to-fiber perimeter exchange (CFPE) index is an estimation of capillary-to-fiber interface, and thus of O_2 diffusion capacity at a fiber-type specific level (Hepple 1997). As shown in Fig. 2C, healthy aging was not characterized by major modifications in CFPE index for any fiber-type. However, hypertension in elderly subjects was associated with a significant decrease in CFPE index for the most abundant fibers (type I and type IIA) and for type IIA-IIX fibers (compare EL and EL-HT in Fig. 2C). MS in elderlies did not further alter CFPE as this index did not differ between the EL-HT and EL-MS subjects.

Although CFPE index estimates O_2 diffusion capacity at a fiber-type level, this index does not take into account the length of contact between capillaries and the fiber. The length of contact to perimeter of fiber (LC/PF) (Vincent *et al.* 2010) was then also used to provide another estimation of the capillary-to-fiber interface. As shown in Fig. 2D and like CFPE index, LC/PF did not differ between healthy YO and EL subjects for any fiber-type. However, hypertension in elderly subjects was associated with a significant decrease in LC/PF for type I and IIA fibers (compare EL and EL-HT in Fig. 2D). Metabolic syndrome in elderly subjects was associated with similar LC/PF reductions for type I and IIA fibers, and with further LC/PF reductions for type IIA-IIX and IIX fibers, when compared to healthy YO and EL subjects. Regression analyses performed for old (EL, EL-HT and EL-MS) men indicated that VO_{2max} correlated positively with LC/PF and CFPE index for type I ($r = 0.55$, $P < 0.023$; $r = 0.59$, $P < 0.006$, respectively) and IIA fibers ($r = 0.61$, $P < 0.004$; $r = 0.58$, $P < 0.007$, respectively).

In addition to CFPE index and LC/PF, capillary exchange capacity can further be modified by capillary tortuosity which is estimated by the CapTor index (Vincent *et al.* 2010). In our study, healthy aging was not characterized by a significant modification in CapTor (YO vs. EL in Fig.3). However, hypertension in the elderly subjects was associated with a significant decrease in this index, as CapTor was signicantly lower for EL-HT when compared with YO, and also tended to decrease when compared with EL ($P \leq 0.1$). MS in elderlies did not further alter CapTor as no significant difference was observed between the EL-HT and EL-MS groups. Therefore, our data suggested that hypertension in elderly, but not aging alone, is associated with a decreased capillary-to-fiber interface in the skeletal muscle.

Increased extracellular matrix area.

Capillaries are embedded inside the extracellular matrix (ECM), and ECM remodeling could be important for capillary-to-fiber exchange and O_2 diffusion capacity (Fig. 4). ECM was then assessed using Sirius red, which labels major ECM components, type I and type III collagens (Tullberg-Reinert & Jundt, 1999), and using image analysis to distinguish ECM endomysium and perimysium. As shown in Fig. 4, healthy aging was mostly associated with an increased perimysium area, while endomysium area did not differ between YO and EL individuals. Interestingly, regression analyses performed for healthy (YO and EL) men revealed that perimysium area was negatively correlated with muscle specific strength ($r = 0.65$, $P < 0.001$).

In contrast, hypertension in the elderly was associated with an increased endomysium area, but with no further change in perimysium area (Fig. 4 EL vs. EL-HT). Metabolic syndrome in the elderly men did not further alter perimysium or endomysium ECM area (compare EL-HT vs. EL-MS in Fig. 4).
Regression analyses performed for old men (EL, EL-HT and EL-MS) indicated that endomysium area correlated negatively with LC/PF index for type I ($r = -0.62$, $P < 0.005$) and type IIA ($r = -0.5$, $P < 0.023$) fibers.

Effect of hypertension and MS on COX activities in old men.
Capillary supply and capillary-to-fiber ratio have been correlated to oxidative capacity or mitochondrial content in skeletal muscle (Poole & Mathieu-Costello 1996; Charifi *et al.* 2004). To investigate mitochondrial oxidative activity in skeletal muscle, COX enzymatic activity was then measured (Fig. 5). Healthy aging was not associated with a significant change in COX activity, except for type IIX fiber (YO vs. EL in Fig. 5). In contrast, the EL-HT group exhibited a strong decrease in COX activity for type I-IIA and IIA fibers when compared to YO and EL groups. Similarly, old men with metabolic syndrome revealed lower COX activity for all fiber types when compared with YO, and lower COX activity for types I, I-IIA and IIA fibers when compared with the EL group. However, no significant change was observed between EL-HT and EL-MS groups.
Regression analyses performed for old (EL, EL-HT and EL-MS) men indicated that COX activity correlated positively with LC/PF for type I ($r = 0.51$, $P < 0.045$), type IIA ($r = 0.6$, $P < 0.026$) and type IIX ($r = 0.53$, $P < 0.05$) fibers. COX activity also positively correlated with the global C/F for type I ($r = 0.6$, $P < 0.014$) and for type IIA ($r = 0.53$, $P < 0.033$). Moreover, the decline in COX activity in type IIA fiber also correlated with CAF ($r = 0.63$, $P < 0.01$) in old men groups.

Discussion

Hypertension is highly prevalent in elderly subjects and is one of the major components of the metabolic syndrome, which is a cluster of risk factors for cardiovascular diseases and type II diabetes. In the present study, we report that hypertension in the elderly is an important factor for the regulation of capillary supply and capillary exchange capacity in the skeletal muscle.

Hypertension, metabolic syndrome and muscle capillarization in aged men.

Capillary supply can be estimated by two global indices, which are capillary density (CD) and capillary-to-fiber ratio (C/F). So far, no consensus has been reached in the literature about the effects of aging on CD and C/F in the skeletal muscle . While some authors reported lower CD in old muscles (Coggan *et al.* 1992a; Frontera *et al.* 2000), others found no significant difference (Proctor *et al.* 1995; Croley *et al.* 2005; Ryan *et al.* 2006). A decrease in global capillary-per-fiber (C/F) has been described in aged skeletal muscles (Andersen 2003), and at the fiber level, the number of capillaries per fiber (CAF) surrounding type II, but not type I, fibers was found to be lower in aged compared with young individuals (Coggan *et al.* 1992a; Croley *et al.* 2005; Ryan *et al.* 2006). However, several studies have also shown that exercise training improves muscle capillarization (Coggan *et al.* 1992b; Charifi *et al.* 2004), and that no significant difference in CD, C/F and CAF was observed between old and young subjects when physical activity was taken into account (Coggan *et al.* 1992b; Proctor *et al.* 1995; Harris 2005). In agreement with these last observations, in the present study healthy active young and elderly subjects exhibited similar levels of CD, C/F and CAF indices. Our data therefore support the hypothesis that aging alone is not a major determinant for capillary supply in human skeletal muscle.

Noteworthy, we also provide evidences that, in elderly subjects with similar physical activity, hypertension is determinant for capillary supply. Moreover, microvascular rarefaction occurred for all major fiber types, as CAF was reduced for type I, IIA and IIX fibers. Previous findings on hypertension have mainly been deduced from experimental animal models, and they agreed that hypertension in rats is associated with microvascular structural rarefaction in skeletal muscle (Chen *et al.* 1981; Prewitt *et al.* 1982; Hedman *et al.* 2000; Feihl *et al.* 2006). To our knowledge, our data are the first to demonstrate capillary rarefaction in skeletal muscle of elderly subjects with hypertension and/or MS.

A relationship between the capillary supply and VO_{2max} has been described previously in old men subjected to exercice training (Hepple *et al.* 1997). Interestingly, the correlations that we observe between VO_{2max} and muscle capillarization indices (CD, CF, CAF) were established for elderly men affected by various MS components, but exhibiting similar physical activity. While it is clear that other factors (e.g., metabolic adjustments, cardiac output, etc.) play a central role in the regulation of VO_{2max}, our findings suggest that, independently of exercise, the role of the capillary supply to muscle fibers is also important to whole body aerobic function in the global older population.

Hypertension, metabolic syndrome and capillary-to-fiber interface in aged men.

The capillary-to-fiber interface is involved in oxygen supply to the muscle and has been suggested to be a sensitive marker for changes in the capillary network of skeletal muscle (Hepple 1997; Hepple & Mathieu-Costello 2001; Charifi *et al.* 2004). Two capillary indices

are generally used to estimate capillary-to-fiber interface at a fiber type level: CFPE and LC/PF. The capillary-to-fiber perimeter exchange (CFPE) index represents the ratio between the capillary-to-fiber ratio calculated for each individual fiber and the perimeter of the fiber. However, a major disadvantage of the CFPE index is its insensitivity to the length of capillaries in contact with the fiber. Therefore, we have also determined another index for assessment of capillary-to-fiber interface (LC/PF) which is based on the length of capillaries, the number of capillaries and the perimeter of the fiber (Sullivan & Pittman 1987; Charifi *et al.* 2004; Charles *et al.* 2006). Although CFPE and/or LC/PF index have been often used to investigate muscle capillary exchange capacity in response to exercise training in elderly (Coggan *et al.* 1992b; Charifi *et al.* 2004; Charles *et al.* 2006), there are only two studies that compared CFPE index between young and aged muscles, and no significant change was found at a fiber-type level (Croley *et al.* 2005; Ryan *et al.* 2006). Our current data confirmed these observations about aging and further established that hypertension in elderly subjects is associated with a significant decrease in CFPE and LC/PF indexes for the most abundant fibers. Of note, hypertension also appeared as the most important amongst MS components, as MS in the elderly only marginally further altered LC/PF. To our knowledge, the present report is the first to demonstrate a lower capillary exchange capacity in aged men with hypertension, suggesting that overall capillary loss would have detrimental effects on muscle diffusive capacity. The positive correlations that we observe between CFPE and LC/PF indexes and VO_{2max} are consistent with a significant role of muscle capillarization supply for whole body aerobic function.

Regardless of capillary density and capillary-to-fiber interface , capillary geometry, caused by branching and tortuosity, is another important factor which may increase surface area contacts between capillaries and muscles. Indeed, microvessels do not simply run straight along muscle fiber, but form sinuous pathways (Janacek *et al.* 2011). Several studies on the microvascular network in skeletal muscle demonstrated that capillary tortuosity change in response to exercise training in elderly (Charifi *et al.* 2004) or in response to pathologies such as α-thalassemia (Vincent *et al.* 2010). However, the importance of aging and/or associated diseases for capillary tortuosity was not previously investigated. A recent study has developed a capillary tortuosity index (CapTor) that takes into account capillary surface area and capillary outer diameter (Vincent *et al.* 2010). Our present results indicated that aging alone was not characterized by major modification in CapTor, while hypertension in the elderly subjects was associated with a significant decrease in this index. Among other MS components hypertension, again, contributed for the most part to the CapTor decrease. By reducing the size of capillary-to-fiber interface, the lower CapTor in hypertensive subjects may constitute another mechanism that contributes to alteration of muscle O_2 diffusion capacity.

Extracellular matrix and its association with capillary supply and exchange capacity. In addition to morphological changes in capillaries caused by tortuosity, the extracellular matrix (ECM) may play an important role in transcapillary exchange (Reed & Rubin 2010). Indeed, ECM is a storage space for interstitial fluid filtered across the capillary wall, and ECM could reduce interstitial fluid pressure (P_{if}) to increase transcapillary fluid flux (Aukland & Reed 1993). For example, the release of the collagen network, may allow normally

underhydrated glycosaminoglycan to expand and take up fluid (Reed & Rubin 2010). Previous studies in rodents have associated stiffness and atrophy of the old muscle with increased ECM collagen concentration and cross-linking (Monnier *et al.* 2005; Gao *et al.* 2008; Ramaswamy *et al.* 2011; Wang *et al.* 2013). However, despite the importance of the muscle ECM network, relatively little information is available on changes in muscle ECM in humans. Previous measurements of intramuscular endomysium collagen in human indicated that it was unchanged with aging (Babraj *et al.* 2005; Haus *et al.* 2007). Herein, we confirm this observation about the endomysium and use image analyses to further demonstrate that healthy aging is nevertheless associated with an increase in perimysium area. The perimysium is thought to be involved in the coordination of shape change in muscle contraction (Purslow 2002), and more perimysium might be important to preserve muscle shape despite age-related fibre atrophy.

Importantly, we also demonstrated that hypertension in old men is associated with an increased endomysium area, while the perimysium remained unaffected. Taken together, a greater muscle endomysium area in hypertensive elderly subjects could be associated with an accumulation of collagen cross-linking, and thus, an increased ECM compaction. Therefore, we could speculate that more endomysium compaction may increase the interstitial fluid pressure, which could contribute to decrease the transcapillary exchange. Consistent with this, we have shown a negative correlation between endomysium area and LC/PF index for the most abundant fiber types (type I and IIA fibers).

Interrelationship between capillary supply and oxidative capacity in aged men with hypertension or MS.

Mitochondria are the main producers of cellular energy. Declines in mitochondrial oxidative capacity were reported with advancing age (Short *et al.* 2005), although physical activity, rather than chronological age, was also reported to be the primary determinant (Peterson *et al.* 2012). We measured cytochrome c oxidase (COX) activity, an inner mitochondrial membrane-embedded enzyme, to estimate oxidative metabolism. While aging did not dramatically affect the global COX activity in skeletal muscle, hypertension and MS were associated with a decreased COX activity in aged muscles.

In human, some studies have investigated the relationship between oxidative capacity or mitochondrial content and capillary supply (Sullivan & Pittman 1987; Poole & Mathieu-Costello 1996; Charifi *et al.* 2004). In the present study, a strong correlation was observed between the index of capillary-to-fiber interface (LC/PF), the number of capillary per fiber (C/F) and the cytochrome c oxidase activity. Therefore, our findings suggests that a decrease in capillary supply, associated with a lower diffusion capacity between the capillaries and fibers, likely contributes to reduce oxidative capacity to muscle fibers, and thus probably induces perturbations in muscle functionality. These conclusions are in line with a recent study showing that old persons who reported greater difficulty with daily activities had lower capillary density and lower oxidative enzymatic activity (Nicklas *et al.* 2008). However, the entire decrease of oxidative metabolism cannot be explained solely by reduced microvascularization, as some mitochondrial dysfunctions such as mitochondrial DNA mutations or accumulation of reactive oxygen species (ROS) also likely implicated in the age-related decline of muscle functionality (Peterson *et al.* 2012). .

In conclusion, our findings clearly demonstrated a capillary rarefaction associated with a decrease of capillary-to-fiber interface and tortuosity in skeletal muscle of elderly subjects with hypertension. Moreover, a relationship between indices of capillary supply and COX activity was observed, and extracellular matrix area negatively correlated with capillary exchange capacity. However, no significant change was observed when hypertensive elderly men were compared to old men with metabolic syndrome. Collectively, our results suggest that hypertension plays a central role in perturbations of skeletal muscle capillarization in aging, and that the other components of metabolic syndrome do not contribute to major additional changes in the aged muscle capillary network.

Experimental Procedures

Ethical approval.

The present study (ClinicalTrials.gov Identifier: NCT00759304) was approved by the Medical Ethics Committee of the University Hospital of Saint-Etienne (France), and was performed in accordance with the principles of the revised Declaration of Helsinki. All subjects were fully informed regarding study participation, and they provided written informed consent.

Subjects.

Our study included 15 healthy young (YO) men, 8 healthy elderly (EL) men, 7 hypertensive elderly (EL-HT) and 9 elderly men diagnosed with metabolic syndrome (EL-MS) for at least 10 years. Subjects were recruited in Saint-Etienne, in France, and old men (heathly, hypertensive and with MS) were selected from the PROgnostic indicator OF cardiovascular and cerebrovascular events (PROOF) cohort which is a prospective longitudinal cohort study of 1011 subjects (Barthelemy *et al.* 2007). Exclusion criteria were prior myocardial infarction or stroke, heart failure, atrial fibrillation, type 2 diabetes, morbid obesity (BMI > 35kg/m^2), Parkinson's disease, and any other disease limiting life expectancy to less than 5 years. Dependant elderly subjects or those living in institution were also excluded. YO subjects were healthy volunteers with the same exclusion criteria than the elderly subjects of the PROOF cohort. All subjects underwent standard medical examination, standard blood analyses (fasting glucose, HDL cholesterol, triglycerides) and performed a maximal stress exercise before their inclusion in the study.

Definition of the metabolic syndrome (MS) and hypertension.

MS was defined according to the National Cholesterol Education Program Adult Treatment Panel III (Grundy 2007), and was diagnosed when 3 of 5 components occurred: waist circumference > 102 cm, triglycerides > 1.7 mmol/l, HDL cholesterol <1.03 mmol/l, blood pressure >130/85 mm Hg, and/or fasting glucose > 5.6 mmol/l (Alberti *et al.* 2009). Blood pressure was measured with the subjects in seated position, and the subjects were selected for the hypertensive group when mean blood pressure was above 140 mmHg systolic and/or above 90 mmHg diastolic, and provided that clinical examination and routine laboratory exams did not show any other diseases.

Daily energy expenditure (DEE), body composition, VO_{2max} and specific strength.

PROOF subjects completed a DEE questionnaire confirming that old men (EL, EL-HT and EL-MS) were physically active. DEE was assessed by a self-administered physical activity questionnaire, with specific emphasis on autonomy and perceived exertion, as previously described (Garet *et al.* 2004). DEE is the sum of n activities in the day and DEE was calculated from the questionnaire using the equation: DEE (kJ/kg/day) = Σ^n (IA_i DA_i), where IA_i is the intensity (in J/min/kg), and DA_i the duration (in min/day) of the activity i.

Body composition was measured using dual X-ray absorptiometry (DEXA, Hologic QDR-2000, Bedford, MA). VO_{2max} was estimated using a cycle ergonometer as previously described (Garet *et al.* 2004). Measurements of maximal knee extension isometric strength and specific strength were performed as described (Verney *et al.* 2006), using a Cybex II

(Ronkonkoma, NY, USA). The knee extensors muscles mechanical response was recorded with a strain gauge (FN3030,0-2000N, FGP Sensor, Les Clayes sous Bois, France) located at the level of the external malleolus. All measurements were taken from the subject's right leg subsequently biopsied, with the knee and hip flexed at 90 degrees from full extension. The volume of the right leg was estimated according to (Jones & Pearson 1969).

Histochemical preparation.

Needle biopsies were taken from the right *vastus lateralis* muscle under local anesthesia in the morning after an overnight fast. Biopsies were mounted with tissue freezing medium, frozen in isopentane cooled on liquid nitrogen and stored at -80°C. Serial cross-sections (10 µm thick) were performed using a cryostat (Microm International, Francheville, France) at -25°C.

Immunohistological assays for fiber type, cross-sectional area (CSA) and capillaries.

Two serial cross-sections were labeled with monoclonal antibodies against myosin heavy-chain-I (MHC-I) (A4.951 from Enzo Life Sciences, Villeurbanne, France) or MHC-IIa (N2.261, Enzo life Sciences), and co-labeled with anti-laminin-α1 (Sigma, Saint-Quentin-Fallavier, France) to outline the fibers, and resolved with corresponding secondary antibodies conjugated to Alexa-Fluor 488 or 546 (Invitrogen, Cergy-Pontoise, France). Images were captured with a high-resolution cooled digital DP-72 camera coupled to a BX-51 microscope (Olympus, Rungis, France) at a resolution of 0.64 µm/pixel. Five fields, each containing 80 fibers, were analyzed per subject. The contractile type (I, I-IIA, IIA, IIA-IIX or IIX), CSA and perimeter were determined for each fiber, using the image processing software Visilog-6.9 (Noesis, Gif-sur-Yvette, France) as previously described (Meunier *et al.* 2010). Thresholding of MHC images distinguished positive, negative or intermediate MHC-labeling. Type-I fibers were positive only for MHC-I, and type-IIA fibers positive only for MHC-IIa. Type-X fibers were negative for both MHC-I and MHC-IIa. Type I-IIA hybrid fibers were intermediate for both MHC-I and MHC-IIa, while type IIA-IIX hybrid fibers were only intermediate for MHC-IIa labeling.

The identification of capillaries was performed using the monoclonal antibody CD31 (M0823 from Dako, Glostrup) which recognizes PECAM-1 (platelet endothelial cell adhesion molecule), a trans-membranous glycoprotein strongly expressed by vascular endothelial cells, as previously described (Charifi *et al.* 2004). Cross-sections were labeled with CD31 antibody, co-labeled with anti-laminin-α1, and resolved with corresponding secondary antibodies conjugated to Alexa-Fluor 488 or 546.

Cytochrome c oxidase (COX) activity and extracellular matrix staining.

COX activity, an index of oxidative capacity, was determined histochemically as previously described (Rezzani *et al.* 2001). Briefly, serial muscle cross-sections were incubated for 1 h at 37 °C with 0.05% diaminobenzidine (D-5637, Sigma) in 0.05 M phosphate buffer (pH 7.3), and then dehydrated and mounted with Eukitt (CML, Nemours, France) and xylene (Elvetec Services, Pusignan, France). COX and MHC images were matched using the Visilog-6.9 software and COX optical density was quantified for each fiber.

For the identification of the interstitial connective tissue (epimysium, perimysium and endomysium), frozen muscle cross-sections were fixed for 1 h in 100 % acetone and stained

for 10 min with Picro-formaline solution, containing 7.5 % picric acid (S70452-498, Sigma) and 4.5 % formaldehyde (F1635, Sigma) in 95 % ethanol buffer. After rinsing in 90 % ethanol for 1 min and in deionized water for 10 min, cross-sections were stained for 1 h with 0.1% picrosirius red, incubated for 5 min in 0.01M HCl solution, and then dehydrated and mounted with Safe Mount (006.47520, Labonord). Sirius red stains ECM red and fibers yellow, and provides important contrast between fibers and ECM, well suitable for image analysis. Image acquisitions were obtained in the bright-field mode, and image analyses were performed using Visilog-6.9 software. The green component of the initial image was used for higher contrast. Top-hat filtering followed by manual thresholding on gray level allowed segmentation of the connective tissue network (perimysium and endomysium). Measurement of the area of this network was performed by counting the number of pixels in the resulting binary images and was expressed as percent of the total field area.

Measurement of capillary network

Global indices. Capillary density (CD) was expressed as the number of capillaries counted per square millimeter (cap.mm^{-2}), in a given area. Capillary-to-fiber ratio (C/F) was calculated as the ratio between the number of capillaries present in an area and the number of fibres in the same area.

Individual fiber indices. For each fiber type, the mean number of capillaries around a single fiber (CAF) was calculated. To estimate the capillary-to-fiber interface, and thus estimate exchange capacity between capillaries and a muscle fiber, the CFPE index (capillary-to-fiber perimeter exchange) was determined as previously described (Hepple 1997; Charifi *et al.* 2004). Briefly, for each fiber, capillaries in contact with that fiber were counted by taking account their sharing factor to determine the capillary-to-fiber ratio for each individual fiber (C/F_i). The CFPE index was then calculated as the ratio between C/F_i and fiber perimeter. The capillary-to-fiber interface was also estimated by LC/PF index, which is the length of contact (LC) between capillaries and the fibers divided by perimeter of the same fiber (PF). LC/PF, originally called "length of capillary-fiber contact", is expressed as a percentage of muscle fiber perimeter in contact with the capillary wall and gives information on the diffusion capacity between the capillary network and the muscle fibers.

Tortuosity. The microvascular network tortuosity was assessed using the following equation: $CapTor = CSA/\pi(COD/2)^2$, where CSA is capillary surface area and COD is capillary outer diameter (Vincent *et al.* 2010). The more the ratio is elevated, the more the capillary is tortuous. A ratio of 1 describes a microvessel that is perfectly perpendicular to the muscle section.

Statistical analysis.

Values are means $\pm$ SE. YO, EL, EL-HT and EL-MS groups were compared using one-factor analysis of variance (ANOVA), followed by post-hoc Fisher's test for pairwise comparisons between groups. Univariate linear regression analysis determined relationships between variables of interest in either lean (YO and EL) or old (EL, EL-HT and EL-MS) populations. Statistical analyses were performed using XLSTAT (Addinsoft, Paris, France) and significance was set at $P < 0.05$.

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Author contributions

M.G., C.C.G. and D.B. contributed to the conception and design of the research; J.V., L.F., F.R. performed clinical research. M.G., C.C.G. and L.T. performed the experiments; M.G., C.C.G. and D.B. analyzed the data; M.G., C.C.G. and D.B. interpreted the results of the experiments; M.G. and L.T. prepared the figures; M.G. and D.B. drafted the manuscript; M.G., C.C.G., L.T., B.M., L.B., D.T., C.P., D.A., B.P., J.V., F.R., L.F., and D.B. edited, revised and approved the final version of the manuscript.

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Figure legends

Fig. 1 Global indices of capillarization in skeletal muscle of young (YO), healthy old men (EL) and elderly with hypertension (EL-HT) or with metabolic syndrome (EL-MS). (A) Capillary density and (B) capillary-to-fiber ratio were determined for all groups. All values in the graphs are means ± SEM (vertical bars) and different letters indicate significant difference ($P < 0.05$) between groups.

Fig. 2 Muscle capillarization at fiber type level in young (YO), healthy old men (EL) and elderly with hypertension (EL-HT) or with metabolic syndrome (EL-MS). (A) Representative images of serial cross-sections labeled (green) for either myosin heavy-chain I (MHC-I) or MHC-IIA, and for capillaries using an anti-CD31 antibody. Cross-sections were counter-stained for laminine-α-1 (red) to outline the fibers. Thresholding of MHC images distinguished either positive, negative or hybrid MHC-labeling. Type I fibers (¶) and type IIA (^) fibers are indicated. (B) Capillary around fiber (CAF), (C) capillary-to-fiber perimeter exchange (CFPE) and (D) length of capillary contact to perimeter fiber (LC/PF) were measured for 400 fibers per subject. All values in the graphs are means ± SEM (vertical bars) and different letters indicate significant difference ($P < 0.05$) between fiber types. * $P < 0.05$ *vs*. YO group. § $P < 0.05$ *vs*. EL group.

Fig. 3 Capillary tortuosity in skeletal muscle of young (YO), healthy old men (EL) and elderly with hypertension (EL-HT) or with metabolic syndrome (EL-MS). (A) Representative images of muscle cross-section labeled for capillaries using an anti-CD31 antibody. (B) Index of capillary tortuosity (CapTor) was determined for 100 capillaries per individual. All values in the graphs are means ± SEM (vertical bars) and different letters indicate significant difference ($P < 0.05$) between groups.

Fig. 4 Extracellular matrix area in skeletal muscle of young (YO), healthy old men (EL) and elderly with hypertension (EL-HT) or with metabolic syndrome (EL-MS). (A) Representative images of muscle cross-sections stained with Sirius Red indicating endomysium (arrow) and perimysium (arrowhead). (B) Endomysium and (C) perimysium area were assessed for 400 fibers were analyzed per individual. All values in the graphs are means ± SEM (vertical bars) and different letters indicate significant difference ($P < 0.05$) between groups.

Fig. 5 Cytochrome c oxidase (COX) activity in skeletal muscle of young (YO), healthy old men (EL) and elderly with hypertension (EL-HT) or with metabolic syndrome (EL-MS). (A) Representative images of serial cross-sections labeled for myosin heavy-chain I (MHC-I), MHC-IIA, and COX activity. MHC labeling (green) was counter-stained for laminin-α1 (red) and used to determine fiber type in YO, EL, EL-HT and EL-MS. Type I fibers (¶) and type IIA (^) fibers are indicated. (B) COX activity was measured for 400 fibers per individual. All values in the graphs are means ± SEM (vertical bars) and different letters indicate significant difference ($P < 0.05$) between fiber types. * $P < 0.05$ *vs*. YO group. § $P < 0.05$ *vs*. EL group.

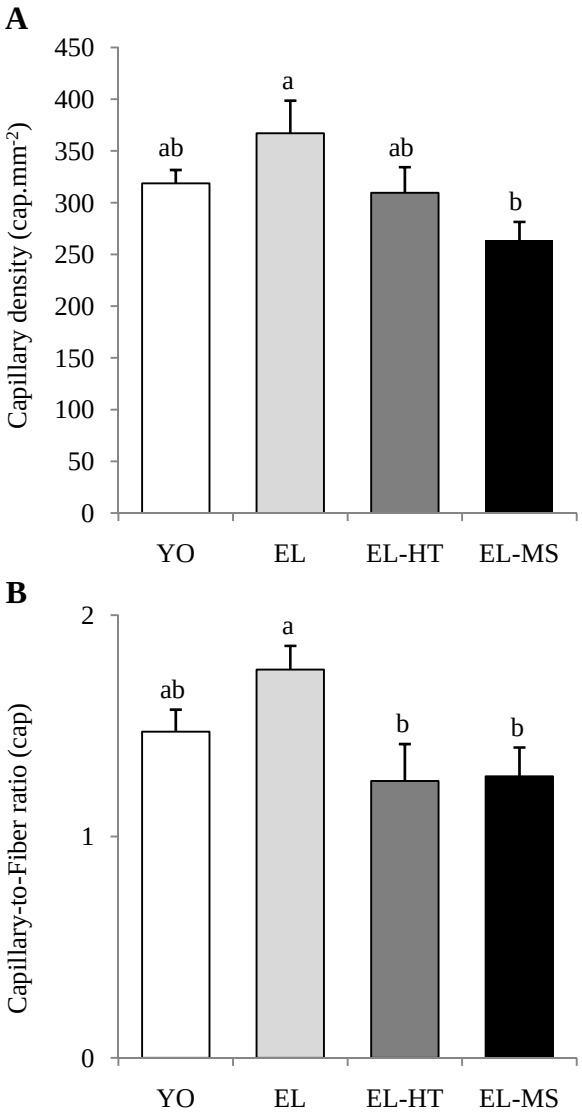

A
Capillary density (cap.mm-2)
450
400
350
300
250
200
150
100
50
0
a
ab
ab
b
YO
EL
EL-HT
EL-MS
B
Capillary-to-Fiber ratio (cap)
2
1
0
a
ab
b
b
YO
EL
EL-HT
EL-MS

Figure 1

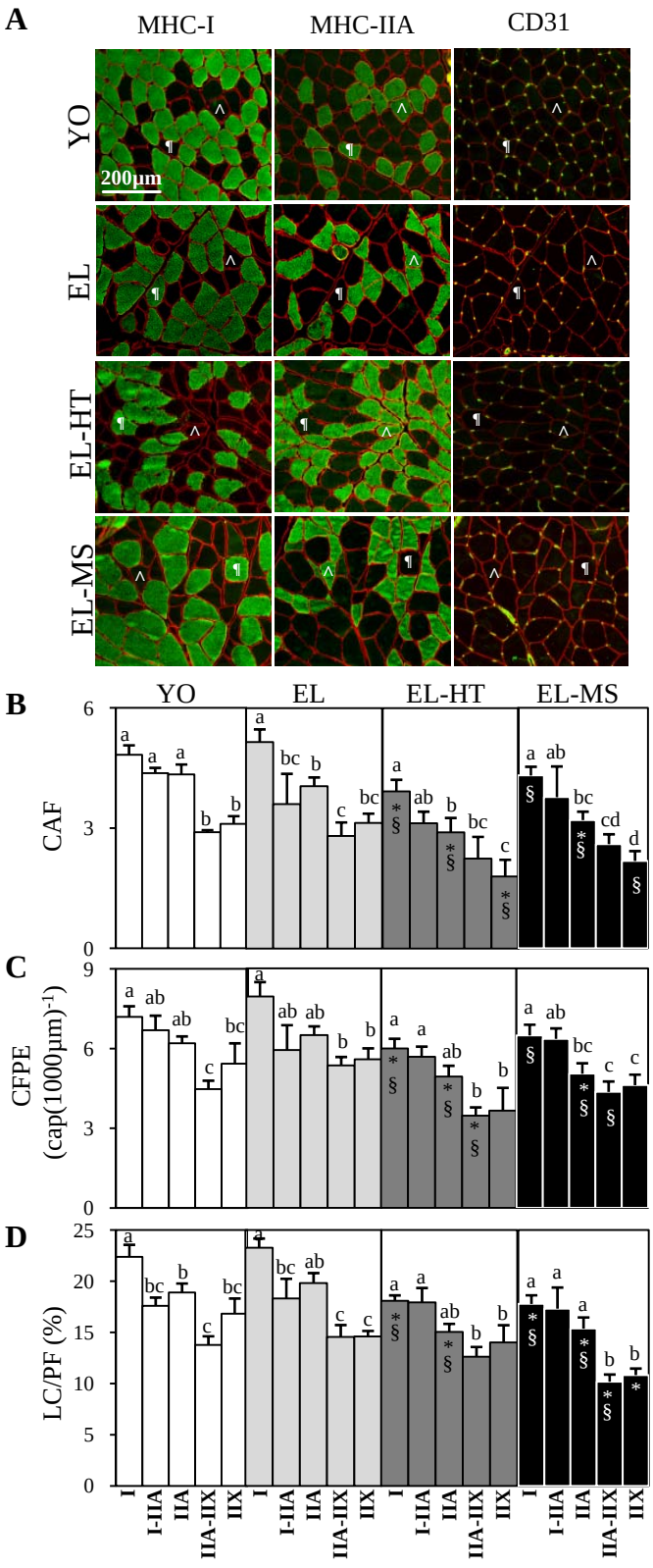

A
MHC-I
MHC-IIA
CD31
YO
EL
EL-HT
EL-MS
200µm
B
C
D
CAF
CFPE
(cap(1000µm)-1)
LC/PF (%)
YO
EL
EL-HT
EL-MS
I
I-IIA
IIA
IIA-IIX
IIX

Figure 2

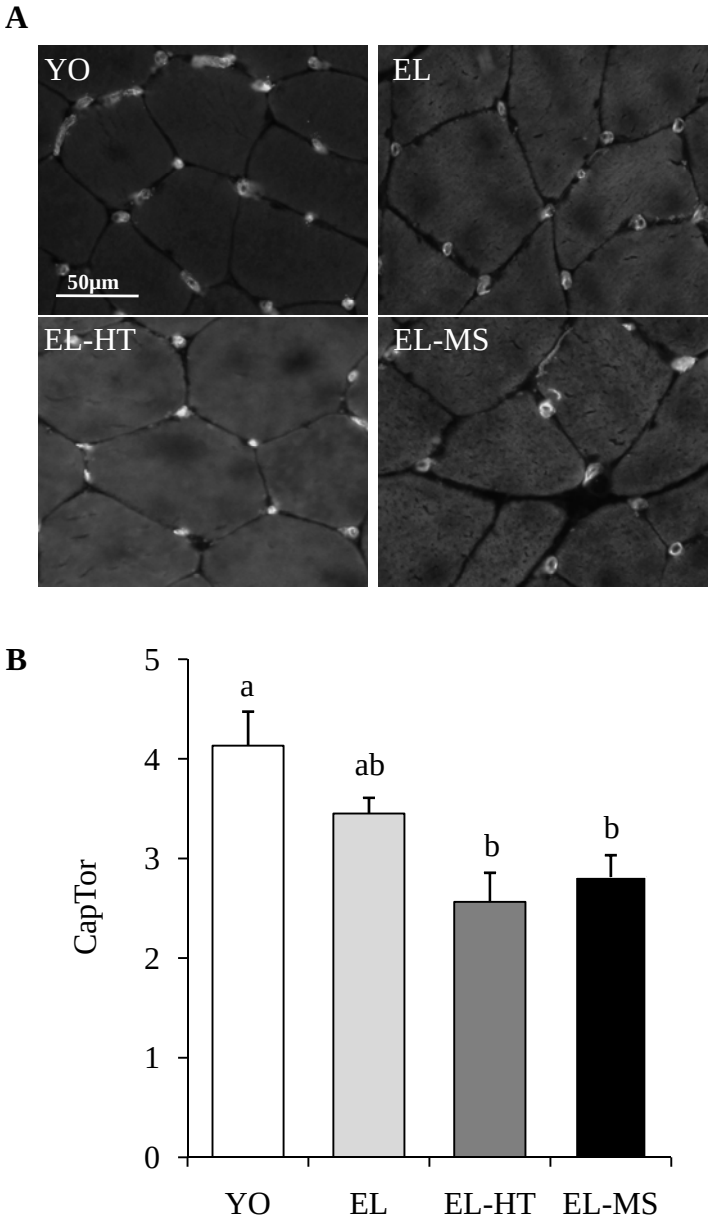
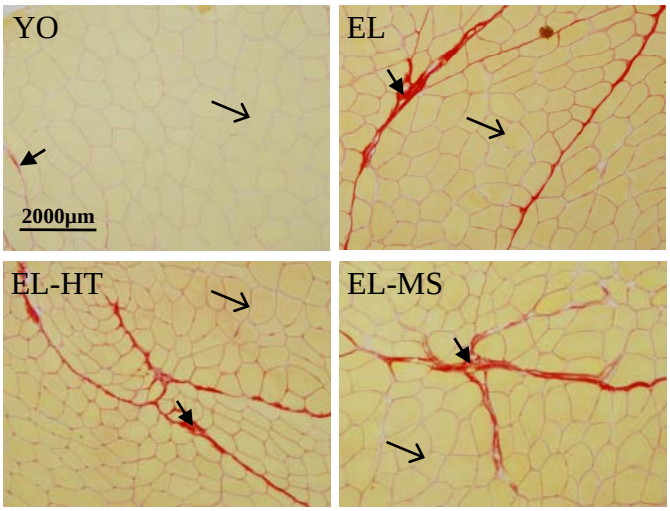


Figure 3

A


YO
EL
2000µm
EL-HT
EL-MS

B

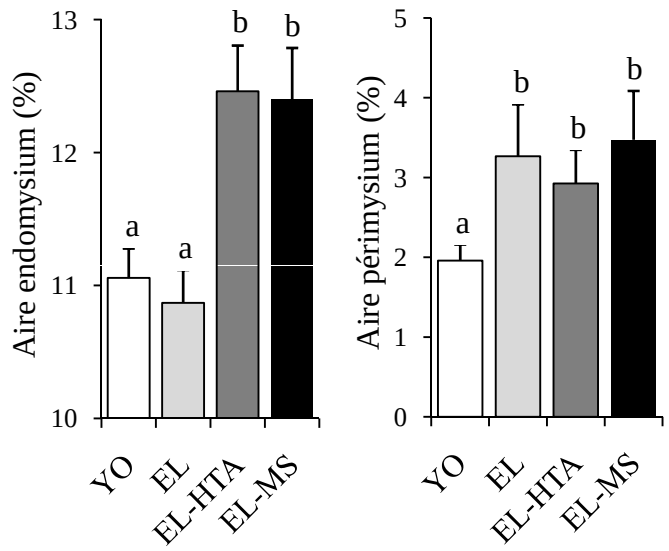

Aire endomysium (%)
13
12
11
10
a
a
b
b
YO
EL
EL-HTA
EL-MS
Aire périmysium (%)
5
4
3
2
1
0
a
b
b
b
YO
EL
EL-HTA
EL-MS

Figure 4

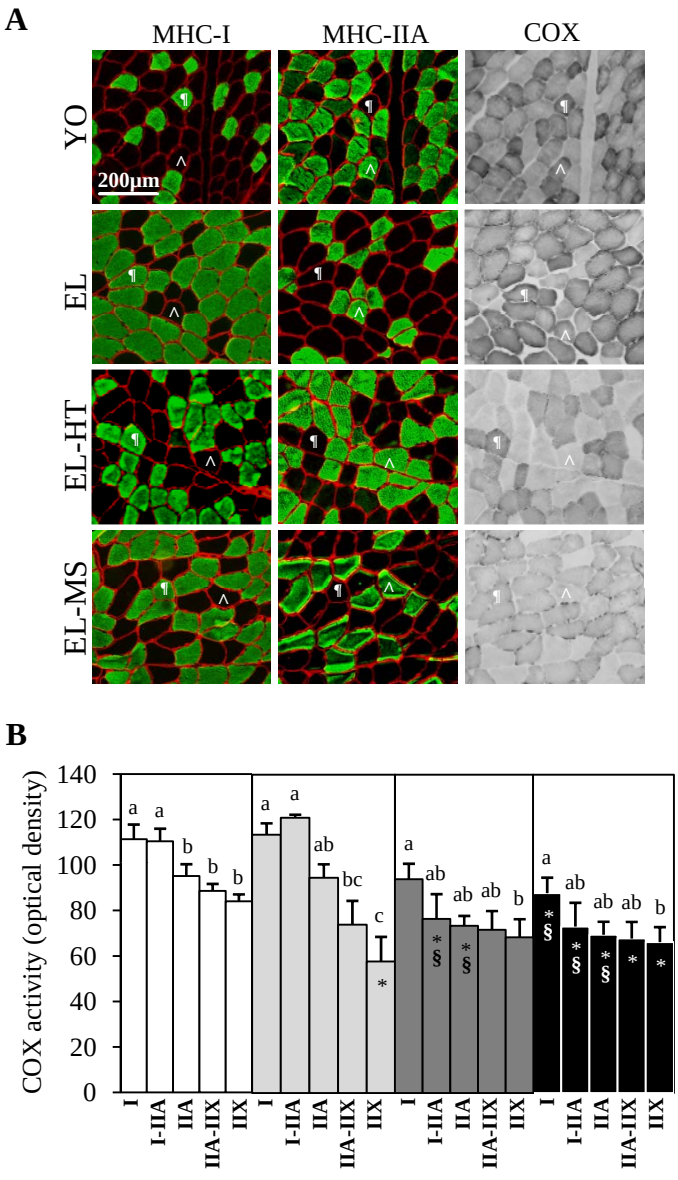

A
MHC-I
MHC-IIA
COX
YO
EL
EL-HT
EL-MS
200µm
B
COX activity (optical density)
0
20
40
60
80
100
120
140
I
I-IIA
IIA
IIA-IIX
IIX

Figure 5

Publication 5

Marine Gueugneau, Cécile Coudy-Gandilhon, Christophe Chambon, Lydie Combaret, Daniel Taillandier, Cécile Polge, Didier Attaix, Brigitte Picard, Frédéric Roche, Léonard Féasson, Daniel Béchet.

“Proteomics of muscle aging in men with or without metabolic syndrome”

En cours de préparation

Proteomics of muscle aging in men with or without metabolic syndrome

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ABSTRACT :

Skeletal muscle aging is characterized by a progressive decline in muscle mass and function, which is referred to as sarcopenia. Aging is also a primary risk factor for metabolic syndrome (MS), which is a cluster of risk factors for cardiovascular diseases and type 2 diabetes. However, the molecular mechanisms implicated in sarcopenia and changes in muscle proteome associated with MS in elderly men remain unclear. Thus, we have undertaken a top-down differential proteomic approach to identify novel biomarkers and total protein extracts were prepared from *vastus lateralis* muscle biopsies of young and old men with or without metabolic syndrome. Overall 586 protein spots were matched between all individuals, and 78 were differentially expressed and identified by LC-MS/MS, corresponding to 43 different proteins. Our results indicate that aging was associated with lower levels of glycolytic enzymes (i.e. glyceraldehyde-3-phosphate, β-enolase, triosephosphate isomerase), with a fast-to-slow transition (ex, slow and fast troponin) and with an up-regulation of several proteins involved in cytoproctection/cytodetoxification (i.e. aldehyde dehydrogenase, HSPA9/A1A/B6, carbonic anhydrase 3) and membrane repair (TRIM72, PTRF). In elderly men, MS was associated with further decrease in the glycolytic metabolism and a fast-to-slow transition seems to be observed. However, MS was also characterized by perturbations in lipid metabolism (i.e. acyl-CoA dehydrogenase, FABP3) and by increased levels of several components of ubiquitin-proteasome system (i.e. proteasome subunit alpha type-1 and beta type-4). Most of the candidate proteins identified herein by differential proteomics were previously unrecognized in human aging skeletal muscle and constitute potential new biomarkers of sarcopenia and metabolic syndrome.

KEYWORDS : Skeletal muscle, aging, old men, proteomics, metabolic syndrome.

▪ INTRODUCTION

The skeletal muscle is one of the most affected organs by aging. Between the ages of 20 and 80 years, the cross-sectional area of the *vastus lateralis* muscle may be reduced by up to 40%[1, 2]. This progressive decline in muscle mass and function, which is also referred to as sarcopenia, contributes to both loss of autonomy, increased prevalence for falls, decreased resistance to metabolic aggression, and thus increased morbidity[3]. Aging is also a primary risk factor for metabolic syndrome (MS), which is a cluster of metabolic and cardiovascular symptoms that increase the risk for type 2 diabetes, cardiovascular diseases, and mortality from all causes[4]. MS components include abdominal obesity, atherogenic dyslipidemia, elevated glucose, a prothrombotic and proinflammatory state, and an elevated blood pressure[5].

Human aging is associated with a general impairment of structural and functional elements of the musculoskeletal system. Indeed, in aged skeletal muscle, several studies have reported muscle fiber atrophy and deformation[1, 6, 7], motor units denervation[8, 9], decreased muscle regeneration capacity[10, 11] and change in extracellular matrix properties[12-14]. Alterations in mitochondria have been also noted in aging, including decreased total volume, increased oxidative damage, and reduced oxidative capacity[15, 16]. Moreover, skeletal muscle is a major site of fatty acid oxidation and insulin-mediated glucose disposal, and dysregulation of intramyocellular lipid metabolism is strongly related to insulin resistance in old men[17, 18]. Hence, the age-dependent loss of muscle mass may trigger severe metabolic side effects potentially important for the development of MS and ultimately type 2 diabetes during aging. Although the overall functional, structural and biochemical alterations in aging muscle have been extensively studied, the molecular mechanisms implicated in sarcopenia remain unclear and changes in protein expression levels associated with MS in elderly men are unknown. To better understand these molecular mechanisms, analyses of the expression profile of muscle proteins in well-defined cohorts of elderly subjects are required.

Previous proteomics analysis of aged skeletal muscle has demonstrated substantial alterations in muscle proteins involved in key metabolic pathways, myofibrillar remodeling, cytoskeleton organization and mechanism of cytoprotection and cytodetoxification[19-22]. However, these studies were focused on animal models of aging, such as the senescent Wistar rat, and few studies have been conducted in human muscle. A previous proteomic survey of human muscle aging has investigated differences in protein expression between young adults (20-25 years) and aged (70-76 years) individuals, and has reported a fast-to-slow transition associated with a glycolytic-to-oxidative shift[23]. However, these results are contradictory and another proteomic analysis has demonstrated a decrease of oxidative enzymes expression and activity with aging[15]. A decrease of enzymes involved in glycolytic metabolism and an increase of proteins involved in cytoprotection and cytodetoxication have also been reported[23, 24].

In the present study, we carried out a comparative survey of total protein extracts from biopsies of *vastus lateralis* muscle of young or elderly men with or without metabolic syndrome. Alterations in key muscle proteins from limb muscle support the idea of generally

perturbed protein expression patterns during human aging and revealed a differential expression pattern for contractile element, metabolic enzymes, regulation of proteins misfolding and proteolytic system with MS in old men.

▪ EXPERIMENTAL SECTION

Reagents. Acrylamide, bisacrylamide and Hybond^{TM}-P membrane were purchased from Amersham Bioscience/GE Healthcare (Little Chalfont, UK). Immobilized pH gradient (IPG) buffers, 18 cm ReadyStrip IPG strips (pH 5-8), and Electrode Wicks were from Bio-Rad Laboratories (Marnes la Coquette, France), and orthophosphoric acide, ammonium sulphate and ethanol absolute were from VWR (Strasbourg, France). All other chemicals were from Sigma (L'Isle-d'Abeau Chesnes, France). Sequence grade-modified trypsin was purchased from Promega (Charbonnières-les-bains, France) and Luminata^{TM} Western Horseradish peroxidase (HRP) Substrate was from Millipore (Molsheim, France). For immunoblotting, the polyclonal antibodies against F-actin-capping protein subunit alpha-2 (CAPZ2), ankyrin repeat domain-containing protein 2 (ANKRD2), T-complex protein 1 subunit beta (CCT2), myozenin-1 (MYOZ1), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), delta(3,5)-delta(2,4)-dienoyl-CoA isomerise (ECH1), short-chain specific acyl-CoA dehydrogenase (ACADS), carbonic anhydrase 3 (CA3), αB-crystallin (CRYAB), aldehyde dehydrogenase 2 (ALDH2), superoxide dismutase (SOD2) and proteasome subunit alpha type-1 were purchased from Genetex (Irvin, CA). The anti-mouse IgG-HRP, anti-rabbit IgG-HRP and anti-sarcalumenin were from Santa-Cruz biotechnology (Heidelberg, Germany).

Ethical approval. The present study (ClinicalTrials.gov Identifier: NCT00759304) was approved by the Medical Ethics Committee of the University Hospital of Saint-Etienne (France), and was performed in accordance with the principles of the revised Declaration of Helsinki. All subjects were fully informed regarding study participation, and they provided written informed consent.

Subjects. Our study included 15 healthy young (YO) men, 15 healthy elderly (EL) men, and 9 elderly men diagnosed with metabolic syndrome (EL-MS) for at least 10 years. EL and EL-MS men were selected from the PROgnostic indicator of cardiovascular and cerebrovascular events (PROOF) cohort which is a prospective longitudinal cohort study of 1011 subjects[25]. Subjects were recruited in Saint-Etienne, France. Exclusion criteria were prior myocardial infarction or stroke, heart failure, atrial fibrillation, type 2 diabetes, morbid obesity (BMI > 35kg/m^2), Parkinson's disease, and any other disease limiting life expectancy to less than 5 years. Dependant elderlies or those living in institution were also excluded. YO subjects were healthy volunteers with the same exclusion criteria than the elderlies of the PROOF cohort. All subjects underwent standard medical examination, standard blood

analyses (fasting glucose, HDL cholesterol, triglycerides) and performed a maximal stress exercise before their inclusion in the study.

Muscle samples were obtained by surgical biopsy from the vastus lateralis muscle and were immediately frozen in liquid nitrogen and stored at -80°C until used.

Definition of the metabolic syndrome (MS). MS was defined according to the National Cholesterol Education Program Adult Treatment Panel III[26], and was diagnosed when 3 of 5 components occurred: waist circumference > 102 cm, triglycerides > 1.7 mmol/l, HDL cholesterol <1.03 mmol/l, blood pressure >130/85 mmHg, and/or fasting glucose > 5.6 mmol/l [5].

Protein extraction. Total muscle extracts were prepared for each subject, and each individual was assessed separately. Muscle aliquots were homozenized (40mg/mL) in a solubilisation buffer containing 8.3 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (v/v) dithiothreitol, and 2% (v/v) IPG buffer pH 3-10 using a TissueRuptor (Qiagen, Courtaboeuf, France), shaken for 30 min on ice and centrifuged for 30 min at 10,000 X *g*. The supernatants were aliquoted and stored at -20°C until analysis. Protein concentration, determined using the Bradford assay system (Bio-Rad), was 6.5 ± 1.2 mg/mL, 6.7 ± 0.8 mg/mL, and 6.7 ± 0.9 mg/mL for YO, EL and EL-MS extracts, respectively.

Two-dimensional gel electrophoresis (2DGE). For total muscle extracts, 700 μg protein were separated using IPG strips of pH 5-8 range for each individual. For isoelectrofocusing, samples were diluted with rehydration buffer containing 8.3 M urea, 1 M thiourea, 2% (w/v) CHAPS, 0.28% (v/v) dithiothreitol, 2% (v/v) IPG buffer (pH 3-10), and 0.01% (w/v) Coomassie Brilliant blue R-250. The IPG strips were passively rehydrated with 330 μl of this solution at room temperature for 16 h under mineral oil in the PROTEAN IEF Cell system (Bio-Rad) at 20°C, and actively rehydrated using Electrode Wicks loaded onto IPG strips for 6 h at 50 V. During active rehydratation, Electrode Wicks were changed every 2 h. Isolectrofocusing was then performed at 0.05 mA per IPG strip at 50 V for 2 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 8000 V for 6 h and finally 8000 V to achieve 46,000 Vh.

The strips were then equilibrated twice for 15 min with gentle shaking in equilibration buffer containing 6 M urea, 50 mM Tris-HCl buffer (pH 8.8), 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate. Dithiothreitol (1% w/v) was added to the first, and iodoacetamide (5% w/v) to the second equilibration buffer.

Separation according to protein mass was carried out using a Protean Plus DodecaCell system (Bio-Rad) on homogenous 20 cm polyacrylamide gels (11% T, 2.6% C). The equilibrated strips were sealed to the top of the horizontal gel with agarose and subjected to 50 V for 1 h followed by 9 mA per gel until the blue dye reached the bottom of the gel.

Visualization of proteins and image analysis. 2DGE gels were fixed overnight in a solution containing 30% (v/v) ethanol and 2% (v/v) orthophosphoric acid, washed twice for 30min in 2% (v/v) orthophosphoric acid and then transferred to a solution containing 18% (v/v) ethanol, 2% (v/v) orthophosphoric acid and 15% (v/v) ammonium sulphate for 30 min. The gels were stained for 72 h with 0.06% (w/v) Coomassie Blue G-250 added to this last

solution. Gels were scanned using the ImageScanner and LabScan-v.5 software (Amersham Bioscience) and protein spots were analyzed and matched between all gels using Progenesis SameSpot software from Non Linear Dynamics (Newcastle upon Tyne, UK). Proteins with significant changed abundance were picked for tryptic digestion from gels.

Protein identification by mass spectrometry. Excised protein spots from 2DGE gels were distained with 25 mM ammonium bicarbonate, 5% (v/v) acetonitrile for 30 min and twice in 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile for 30 min each. Protein spots were then dehydrated using 100% acetonitrile for 10min and were completely dried using a Speed Vac. Proteins were digested overnight at 37°C using 10 ng/µl of sequence grade-modified trypsin in 25 mM ammonium bicarbonate. Peptide extraction was optimised by adding 100% acetonitrile, followed by 15 min of sonication.

For LC-MS/MS mass spectrometry analysis, peptides mixtures were analysed by on-line nanoflow liquid chromatography using the Ultimate 3000 RSLC (Dionex, Voisins le Bretonneux, France) with 15 cm nanocapillary columns of an internal diameter of 75 lm (Acclaim Pep Map RSLC, Dionex)[27]. The gradient consisted of 4–50% (v/v) acetonitrile in 0.5% (v/v) formic acid at a flow rate of 300 nl/min for 30 min. The eluate (6 ll) was electrosprayed into an LTQVelos (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source. The LTQVelos was operated in a CID top 10 mode (1 full scan MS and the 10 major peaks in the full scan are selected for MS/MS). Raw files were processed using version 1.2 of Thermo Proteome Discoverer. For protein identification, the NCBInr suscrofa protein database (41000 seq) was combined with the sequences of human keratin contaminants. Peptide mass tolerance was set to 1.5 Da and fragment mass tolerance was set to 0.8 Da. Two missed cleavages were allowed. Protein identification was validated when at least three peptides originating from one protein showed significant identification Mascot scores ($P < 0.05$). In the present study we considered that proteins validated for the whole analysed spots were really present on the basis of score, peptide number related to each protein, and MW agreements.

Immunoblotting. For Western-Blot analysis, total protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to Hybon^{TM}-P membrane and probed with anti-CAPZ2, anti-ANKRD2, anti-CCT2, anti-MYOZ1, anti-GAPDH, anti-ECH1, anti-ACADS, anti-CA3, anti-ALDH2, anti-CRYAB, anti-SOD2, and anti-PSMA1 (Euromedex, Souffelweyersheim, France). Primary antibodies were detected with horseradish peroxidase-linked goat anti-mouse or anti-rabbit secondary antibodies, immunoreactive proteins were detected using enhanced chemiluminescence using a CCD camera (GBOX, SYNGENE).

Statistical analysis. Values are means $\pm$ SE. Statistical analyses of each dependent variable (age and MS) were carried out using one-way ANOVA. Multiple comparisons of the honestly significant differences were assessed by Fisher's test. The P value < 0.05 was used as the basis for the conclusion of significant difference.

▪ RESULTS AND DISCUSSION

Differentially expressed proteins during aging with or without MS

In order to evaluate age- and MS-dependent alterations in the skeletal muscle proteome, total protein extracts from muscle biopsies of young versus old men with or without MS were resolved by 2DGE. Thirty-nine gels with medium range IPGs (pH 5-8) were performed to analyze total protein extracts. Each gel was duplicated and 2DGE revealed 586 protein spots that were matched between all individuals. 88 protein spots were found to be differentially expressed and 78 were identified by LC-MS/MS, corresponding to 43 different proteins. Aging was associated with the differential expression of 27 different proteins, and metabolic syndrome with the differential expression of 27 different proteins. Between these 2 conditions, 11 common proteins were found. Among them, 5 varied in a same manner and 6 varied in an opposite manner during aging or MS.

Table 1 summarizes the main properties of the proteins differentially regulated in skeletal muscle between young and old men with or without MS.

Perturbations of the myofilaments networks and cytoskeleton during aging with or without metabolic syndrome

In skeletal muscle, muscle movement depends on interactions between myosin-thick and actin-thin filaments. Myosin is a hexameric protein that consists of two heavy chain subunits, two alkali light chain subunits and two regulatory light chain subunits. Myosin light chains typically present various isoforms, and in our study, muscle aging was associated with higher level of three isoforms of the myosin light chain 6B (MYL6B; spots 917, 1820 and 1851), while MS was associated with higher level of the myosin light chain 2 (MYL2; spot 979). In addition to myosin as its major component, the thick filament contains important regulatory proteins, such as myosin-binding protein C (MYBPC1; spot 1930) which was selectively down-regulated in EL-MS muscles compared to YO and EL groups. MYBPC1 has significant effects on length, thickness and lateral alignment of myosin filaments[28]. A reduction in MYBPC1 has previously been reported in the old skeletal muscle in rat[19], which could thus raised the possibility that low MYBPC1 level may favor decreased contractile capacities in aged muscles with MS.

As previously described in rat and human, muscle aging was further associated with the down-regulation of one isoform of skeletal α-actin (ACTA1; spot 714), and with the differential expression of other major components of the thin filament. Thus, a down-regulation of two isoforms of fast troponin T (TNNT3; spots 754 and 1926) which is a major regulator of the thin filament, and the up-regulation of five isoforms of slow troponin T (TNNT1; spots 1825, 1826, 1859, 1870 and 1873) were found in EL muscle, when compared to YO muscle. Troponin T directly interacts with key components in the thin filaments regulatory system to mediate the activation and force development of actomyosin contractile units[29]. In old men with MS, perturbations in contractile proteins of the thin filament were also observed. In contrast to aging, MS was associated with an increased expression of two isoform of ACTA1 (spots 683 and 714), but further associated with a down-regulation of TNNT3 (spot 1896) and an up-regulation of skeletal muscle troponin C (TNNC2; spot 962).

Troponin C is the Ca^{2+}-binding subunit of the Troponin complex and its interaction with Troponin I and Troponin T is central to the regulation of skeletal muscle contraction[30].

In addition to the troponin complex, the thin filament contains many other proteins which anchor actin filaments across the Z-disk and regulate its growth[31]. In aged skeletal muscles with MS, the expression of two actin-binding proteins, F-actin-capping protein subunit α-2 (CAPZA2; spot 793) and cofilin-2 (CFL2; spot 988), was increased. By capping the barbed end of actin filaments, F-actin-capping protein (CapZ) regulates the growth of the actin filament at the barbed end, and thus has an important role in the regulation of actin dynamic[32, 33]. Cofilin-2 is a small actin-binding protein, and a member of a group of proteins that include cofilin-1, cofilin-2 and destrin, formerly called actin depolymerization factor [34]. These proteins are critical in the regulation of actin filament dynamics in eukaryotes where they function by modulating actin depolymerization and severing. Therefore, cofilin-2 is essential for muscle maintenance. A higher level of CAPZA2 and CFL2 could be associated with the higher expression of two isoforms of skeletal α-actin observed in aged muscle with MS.

Myofibrillar-associated proteins can participate in signal transduction cascades. Aging was thus associated with an increased expression of the ankyrin repeat domain-containing protein 2 (ANKRD2; spot 745). ANKRD2 is a member of the mechano-sensing proteins that link myofibrillar stress response to muscle gene expression and is preferentially expressed in slow type-I fibers[35, 36]. ANKRD2 interacts both with I-band sarcomeric proteins and with nuclear transcription factors[37]. ANKRD2 is also induced by denervation, which is consistent with neuronal remodeling in the old muscle[38].

Western-blotting experiments confirmed the overexpression of ANKRD2 and cofilin-2 in skeletal muscle of young individuals compared to healthy elderly or elderly with MS, respectively (Figure 2).

In all, the present findings reported several modifications in sarcomeric actomyosin and regulatory proteins, which may contribute to the disorganization of myofibres in old muscles. Moreover, a decreased expression of ANKRD2 and TNNT3 in addition to an increased expression of MYL6B and TNNT1 are consistent with a fast-to-slow transition of the old skeletal muscle [39]. Similarly, the down-regulation of TNNT3 associated with an up-regulation of MYL2 and TNNC2 could also reflect a fast-to-slow transition in aged muscle with metabolic syndrome. Moreover, metabolic syndrome was also characterized by perturbations of the myofibrillar network, in particular, in the actin filament. These changes suggest an influence of aging and MS process on the maintenance of the proper organization of sarcomeres in regular structures that are closely linked to costameres, and on the response to extracellular signals. Thus, the age and MS-related changes of skeletal muscles may result in loss of muscle force and contractile speed and contribute to the development of sarcopenia.

Changes in signal transduction in skeletal muscle of elderly men with or without metabolic syndrome

The composition of skeletal muscle, in terms of the relative number of slow- and fast-twitch fibers, is tightly regulated to enable an organism to respond and adapt to changing physical demands. The phosphatase calcineurin and its downstream targets, transcription factors of the nuclear factor of activated T cells (NFAT) family, play a critical role in this process by

promoting the formation of slow-twitch, oxidative fibers[40]. In the present study, we have identified a down-regulation of calsarcin-2, also termed myozenin-1 (MYOZ1; spot 1844), which is expressed in fast-twitch fibers of skeletal muscle and regulates calcineurin/NFAT activity[40]. In contrast, MS was associated with an increased expression of this same myozenin-1 isoform (spot 1844) compared to healthy elderly, and this was confirmed by Western-Blotting experiments (Figure 3). Therefore, while a lower level of MYOZ1 could promote oxidative fibers formation and contribute to the fast-to-slow transition in skeletal muscle during aging, a higher level of MYOZ1 could reduce slow-twitch formation in EL-MS muscles.

In skeletal muscle, intracellular calcium (Ca^{2+}) is an important secondary messenger for signal transduction and is essential for cellular processes such as excitation-contraction coupling. Thus, numerous proteins can bind Ca^{2+}, and muscle aging was associated with lower level of annexin A5 (ANXA5; spot 1901) which binds to phospholipids in the presence of Ca^{2+}. Annexins have been involved in a broad range of molecular and cellular processes, and ANXA5 seems to promote membrane repair by self-assembling into two-dimensional arrays on membranes[41].

Perturbations in the energy metabolism during aging with or without metabolic syndrome

Energy for the regulation and maintenance of the excitation-contraction-relaxation cycle is supplied by ATP via the phosphocreatine shuttle, anaerobic glycolysis, the citric acid cycle and oxidative phosphorylation. Aging is also characterized by disturbance in energy metabolism.

Phosphocreatine shuttle

Creatine/phosphocreatine is central to maintain energetic homeostasis as it catalyses the transphosphorylation between phosphocreatine and ADP in intracellular sites of energy demand, and supplies ATP for the actomyosin contractile unit. Here, perturbations in the phosphocreatine shuttle of old muscle were indicated by the up-regulation of monomeric creatine kinase (CKM; spot 1941), which in consistent with a previous report[24].

Anaerobic glycolysis and NADH shuttle

As expected, in the present study, enzymes involved in anaerobic metabolism have been found to be down-regulated during aging. Amongst these, five isoforms of muscle glycogen phosphorylase (PYGM; spot 330, 341, 350, 353, 355) decreased with aging. PYGM catalyzes the phospholytic cleavage of a glucosyl residue from the glycogen polymer to produce glucose-6-phosphate, the major substrate of glycolysis pathway. Decreased activities of glycolytic metabolism were also previously reported in rat[19, 20] and in human[23, 24] skeletal muscles. Five isoforms of triosephosphate isomerase (TPI1; spots 1842, 1845, 1862, 1876, and 1879), that catalyzes the isomerization of the dihydroxyacetone phosphate (DHAP) and

D-glyceraldehyde-3-phosphate, were down-regulated, while three isoforms (TPI1; spots 234, 963 and 1884) were up-regulated with aging. The D-glyceraldehyde-3-phosphate is then converted to 1,3-diphosphoglycerate by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH; spot 773), which selectively decreased in aged muscle and this was confirmed by Western-Blotting experiments (Figure 4A). Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. In old men, four isoforms of β-enolase (ENO3; spots 677, 748, 1857 and 1874) were down-regulated with aging. Finally, we also detected lower levels of pyruvate kinase isozyme M1/M2 (PKM; spot 561) which catalyzes the last step within glycolysis, the dephosphorylation of phosphoenolpyruvate to pyruvate, and is responsible for net ATP production within the glycolytic sequence.

In elderly, MS was also associated with a down-regulation of several glycolytic enzymes previously identified in aged muscle. Indeed, two isoforms of PYGM (spots 340, 347), three isoforms of TPI1 (spots 1841, 1845, 1846), α-enolase (ENO1; spot 658), two isoforms of ENO3 (spots 672, 1858) and PKM (spot 562) were found to decrease in skeletal muscle of old men with MS. In addition, we also detected lower levels of phosphoglycerate mutase 2 (PGAM2; spot 1908), which catalyze the conversion of the 3-phospho-D-glycerate to 2-phospho-D-glycerate. In human, PGAM2 deficiency has been associated with the presence of sarcolemmal tubular aggregates and myopathy[42]. Moreover, the lactate produced by glycolysis pathway could be converted to glycogen. In old muscle, we have detected a higher level of fructose-1,6-bisphosphatase isoenzyme 2 (FBP2; spot 799), an enzyme which participates in glycogen synthesis from lactate[43, 44]. In contrast, this enzyme was decreased in old men with MS, confirming a reduction in energy metabolism in these individuals.

NADH reducing equivalents not only participate in reduction-oxidation reactions as co-substrates but also play important roles in metabolic regulations in both cytosol and mitochondria[45]. However, the inner mitochondrial membrane is impermeable to NADH and NAD^+, so effective transport of these species between cytosol and mitochondria is accomplished via specialized shuttle systems, such as the malate/aspartate shuttle. Our results show that MS in elderly was associated with a decreased expression of cytosolic aspartate aminotransferase (GOT1; spot 735). This enzyme is a major component of the malate/aspartate shuttle and catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate in the cytosol[46]. In old men, MS-related decline in cytoplasmic GOT1 may indicated reduced oxidation of cytoplasmic NADH and is consistent with the decreased glycolytic enzymes expression observed in the EL-MS group.

Mitochondrial energy and lipid metabolism

Unlike activities of glycolytic enzymes, expression of mitochondrial enzymes is more controversial in the literature[15, 23, 39]. In the current study, no differential protein expression was observed between young and elderly men with or without MS, suggesting that aging weakly impairs oxidative enzyme expression, such as enzymes involved in the citric acid cycle and oxidative phosphorylation. However, differential expression of several mitochondrial enzymes involved in lipid oxidation was here reported.

In addition to glucose, lipids are another source of energy in skeletal muscle. Several intracellular fatty acid binding proteins (FABPs) have been identified. They have important functions in the transport of intracellular fatty acids by increasing their solubility and have been shown to enhance the transport of fatty acids from the cell membrane to the site of oxidation, such as mitochondria, and to the site of esterification into intramyocellular triglycerol[47]. MS in elderly was associated with the up-regulation of the heart FABP (FABP3; spot 1486), which is the major FABP in skeletal muscle and plays a permissive role in fatty acid uptake by skeletal muscle[48 49]. Thus, MS-related increase in FABP3 expression may induce higher lipid content in skeletal muscle.

Furthermore, MS was also associated with a down-regulation of short-chain specific acyl-CoA dehydrogenase (ACADS; spot 1856), which catalyzes the initial step in each cycle of fatty acid β-oxidation in mitochondria[50]. The degradation of unsaturated fatty acids by β-oxidation requires several auxiliary enzymes, such as the delta(3,5)-delta(2,4)-dienoyl-CoA isomerase (ECH1) which catalyzes the isomerization of 3,5-dienoyl-CoA to 2,4 -dienoyl-CoA. An up-regulation of one isoform of ECH1 (spot 839) was observed in skeletal muscle of healthy elderly, while another isoform (ECH1, spot 837) was up-regulated in old men with MS. These results suggest that aging and particularly MS were associated with alterations of mitochondrial β-oxidation. Western blotting experiments have confirmed the differential expression of ACADS and ECH1 in muscle of old men with MS compared to young and healthy elderly, respectively (Figure 4B and C).

In all, our observations provide strong evidences for altered regulation of energy metabolism, in particular in anaerobic glycolysis, which could contribute to reduce ATP production in skeletal muscle during aging. These alterations seem to be raised in old men with metabolic syndrome, for whom a strong decrease of glycolytic enzymes expression was associated with a lower level of GOT1, an enzyme central for NADH shuttle. Moreover, previous studies have reported that dysregulation of intramyocellular lipid metabolism is strongly related to insulin resistance, and thus metabolic syndrome, in old men[17, 18]. Consistent with this, we have reported an up-regulation of FABP3 associated with alterations of fatty acid β-oxidation enzymes, such as ACADS and ECH1.

Cytoprotection and cytodetoxification in old muscle with or without metabolic syndrome

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism that can cause cellular damage by oxidation of lipids, proteins, and nucleic acids. Accumulating evidences suggest that oxidative stress underlies the aging process in skeletal muscle[51]. Dismutation of superoxide anion ($O_2^{\cdot-}$) yields hydrogen peroxide (H_2O_2), which can produce highly reactive hydroxyl radical ($^{\cdot}OH$) by Fenton reaction. Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2, and could prevent insulin resistance by reducing the oxidative damage[52]. Mitochondrial superoxide dismutase (SOD2; spot 928) was down-regulated with MS in old muscle, which could likely contribute to insulin resistant in these

individuals. The removal of H_2O_2 in cells is mediated by several enzymes, such as peroxiredoxin[53]. While aging of the human skeletal muscle was associated with lower levels of peroxiredoxin-2 (PRDX2; spot 937), MS was associated an up-regulation of this enzyme. Western blots have confirmed the down-regulation of SOD2 in elderly with metabolic syndrome compared to healthy elderly (Figure 5A).

Oxidative stress also increases the production of cytotoxic aldehydes. Protection against reactive aldehydes is provided by several families of detoxification enzymes, including aldehyde dehydrogenase. Our results revealed that mitochondrial aldehyde dehydrogenase (ALDH2; spot 1866) was up-regulated with aging. Similar regulations were previously reported during aging in rat skeletal muscle in ALDH2 expression[19, 54], however, no previous study of muscle aging notified the differential expression of ALDH2 in human. Western-blotting experiments confirmed that the old human muscle exhibited higher levels of ALDH2 that young muscle (Figure 5B). In contrast, in old muscle with MS, we have observed a decreased expression of other aldehyde dehydrogenase: the 4-trimethylaminobutyraldehyde dehydrogenase (ALDH9A1; spot 1865), suggesting an increase in reactive aldehydes in these individuals. Toxic aldehydes can also be produced by glycolysis, such as methylglyoxal. As previously described above, TPI1 ensures that DHAP produced by aldolase is further metabolized by the glycolytic enzymes. Impairment of TPI1 may result in conversion of DHAP into toxic methylglyoxal, which is eliminated by glyoxalase system[55, 56]. Glyoxalase domain-containing protein 4 (GLOD4; spot 815) was more abundant in old men. In the aged muscle, increased levels of GLOD4may then also represent a compensatory adaptation to avoid excessive formation of toxic products[57]. Western blots experiments have confirmed the up-regulation of GLOD4 (Figure 5C).

Against cellular stress, quality control of protein folding and homeostasis represents a fundamental cellular activity. The proteomic analysis described here demonstrated an up-regulation of 4 heat shock proteins (HSP) which can be classified in 2 groups: HSPA and HSPB[58]. The HPAs are ATP-dependent chaperones essential for protein homeostasis. They contribute to the folding and assembly of nascent polypeptides, the transport of proteins across membranes, and the selection of misfolded proteins for degradation[59]. Muscle aging was associated with the up-regulation of two HSPA proteins, the heat shock 70 kDa protein 1A/1B (HSPA1A or HSP70; spot 496) and the mitochondrial stress-70 protein (HSPA9 or GRP75; spot 468). Overexpression of HSP1A1 was reported to attenuate muscle atrophy induced by immobilization and lengthening contractions[60, 61]. It is therefore likely that the aged human muscle may increase HSPA1A levels as a protection against muscle atrophy. HSPA9 plays a key role in the folding of matrix-localized mitochondrial proteins and is the only know mitochondrial Hsp70 chaperone[62]. In addition to the increase of these two HSPA, two small heat shocks proteins (HSPB) were up-regulated in old muscle: the heat shock protein β-6 (HSPB6 or HSP20; spot 960) and the alpha-crystallin B chain (CRYAB or HSPB5; spot 958). Moreover, an increased expression of the small heat shock β-1 (HSPB1 or HSP27; spot 893) was also found in old men with MS. These HSPBs are ATP-independent chaperones that prevent the aggregation of improperly folded or partially denatured proteins. Similar increases in HSPB6 and CRYAB were previously described in rat muscle during aging[20, 63-65]. Finally and in addition to the HSP proteins, the chaperonin containing T-complex polypeptide 1 subunit 2 (CCT2; spot 619) was also found to increase with human

aging. CCT2 assists the folding of newly translated polypeptides through multiple rounds of ATP-driven release and rebinding of partially folded intermediate forms. Substrates of CCT include the cytoskeletal proteins actin and tubulin[66]. Therefore, the increase of CCT2 likely contributes to maintain cytoskeletal and myofibrillar integrity. The differential expression of CRYAB and CCT2 between young and old muscle was here confirmed by Western blot experiments (Figure 5D and E).

Finally, our proteomic analysis of muscle aging in men indicated an overexpression of four isoforms of the carbonic anhydrase 3 (CA3; spots 885, 887, 1821, 1822), and this was confirmed by Western blotting (Figure 5F). While no consensus has been reached in the literature about CA3, our shot-gun study in women has confirmed the increased expression in aged muscle[67]. The various isoforms of CAs play a crucial role in the acid-balance, CO_2-removal and CO_2-provision for metabolic processes.

Changes in proteolysis in old men with or without metabolic syndrome

Cellular proteins are constantly degraded and resynthesized. To facilitate this recycling, several complex systems have evolved to maintain a controlled and regulated turnover of cellular proteins. The ubiquitin-proteasome system (UPS) is a major non-lysosomal protein degradation machinery[68]. In muscle, the UPS is required to remove sarcomeric proteins upon changes in muscle activity, and thus plays a critical role in skeletal muscle atrophy[69, 70]. There are two sequential steps in the ATP-dependent UPS. First, a poly-ubiquitin chain of at least four ubiquitins is covalently attached to the substrate protein, and this process involves a cascade of enzymatic factors, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin–protein ligases (E3). Second, the poly-ubiquitin signal is recognized by the 26S proteasome which breaks-down the substrate protein into peptides. The 26S proteasome consists of a 20S proteasome core and two 19S regulatory subunits. Our present proteomic analysis report the up-regulation of two subunits of the 26S proteasome in skeletal muscle of old men with MS: the proteasome subunit alpha type-1 (PSMA1; spot 850), the α6 subunit of the 20S core complex, and the proteasome subunit beta type-4 (PSMB4; spot 1854), the β7 subunit of the 20S core complex. These results suggest that MS is associated in an increased proteolysis in old skeletal muscle, which may contribute to muscle atrophy. Moreover, the differential expression of PSMA1 between healthy elderly and elderly with MS was confirmed by Western blot (Figure 6).

In addition to proteasome subunits, we have also identified a decreased expression of the ubiquitin carboxyl-terminal hydrolase 14 (USP14; spot 563) during aging, while this protein was up-regulated in old muscle with MS. USP14 is a deubiquitinase, which releases ubiquitin from the proteasome targeted polyubiquitinated proteins and which ensures the regeneration of ubiquitin. In a previous report, USP14 was observed to be highly induced on microarray analysis in several catabolic conditions including fasting, uremia, diabetes and cancer[71]. With metabolic syndrome in elderly, USP14 may be upregulated in order to maintain the pool of free ubiquitin required for the increased overall conjugation and degradation of muscle

proteins as well as to regulate the stability and function of proteins that are essential muscle metabolism[72].

Perturbations in membrane repair in old skeletal muscle

Repair of damage to the plasma membrane is an important aspect of cellular physiology, and disruption of this process can contribute to pathophysiology in a number of human diseases, including muscular dystrophy. The cell membrane repair response involves translocation of intracellular vesicles to the injury site to form a membrane repair patch composed of several proteins, including the tripartite motif-containing protein 72 (TRIM72, also called MG53). TRIM72 contributes to intracellular vesicle trafficking and is an essential component of the membrane repair machinery in striated muscle[73]. It has been reported that TRIM72 trafficking to the membrane injury site requires the polymerase I and transcript release factor (PTRF)[74]. PTRF, also known as cavin-1, is enrichment in caveolae and contributes to the stable formation of caveolae[75]. In the study of Zhu et al. (2011), it was shown that PTRF anchors TRIM72 to the acute injury site by binding the cholesterol exposed during damage to the cell membrane. Our present findings reported an up-regulation of TRIM72 (spot 620) and PTRF (spot 1885), suggesting that over-expression of these proteins could protect against damage to plasma membrane in skeletal muscle during aging.

To our knowledge, no previous study of aged muscle has reported the differential expression of TRIM72 and PTRF.

Oxygen transport and metabolic syndrome in aged muscle

Hemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates as well as the tissues of some invertebrates. Hemoglobin in the blood carries oxygen from the respiratory organs (lungs or gills) to the rest of the body where it releases the oxygen to burn nutrients to provide energy to power the functions of the organism, and collects the resultant carbon dioxide to bring it back to the respiratory organs to be dispensed from the organism. In the current study, we identified a differential protein spot between elderly controls and elderly with MS, which decreased and was identified as hemoglobin subunit beta (HBB; spot 2101). As hemoglobin is essential for oxygen supply required for muscle metabolism and function, a decrease in HBB could have a negative impact in muscle functionality. Lower levels of HBB may relate with impairments in blood-flow distribution that have been described in the old skeletal muscle [76].

▪ CONCLUSION

Our proteomic analysis revealed important changes in old skeletal muscle with or without metabolic syndrome. During aging, a decrease in the glycolytic metabolism was associated

with a fast-to-slow transition and with an up-regulation of several proteins involved in cytoproctection/cytodetoxification and membrane repair. In elderly men, MS was also associated with a decrease in the glycolytic metabolism and a fast-to-slow transition was also apparent. In aged muscle with MS, our findings also support perturbations of lipid metabolism and increases in components of the ubiquitin-proteasome system. Most of the candidate proteins identified herein by differential proteomics were previously unrecognized in human aging skeletal muscle. To our knowledge, this is the first proteomic analysis investigating muscle proteome changes associated with metabolic syndrome in old men. Identified proteins indicate potential mechanisms of aging and lead to development of biomarkers that may be targets for the comprehension, prevention and treatment of sarcopenia and metabolic syndrome.

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Table 1. Identification of differencially expressed protein in vastus lateralis muscle of young (YO), healthy elderly (EL) and elderly with metabolic syndrome (EL-MS).

# Spot	Accession	Gene	Protein	Anova (p)	Fold change EL vs YO	Fold change EL vs EL/MS	Fold change EL/MS vs YO	Score	% Coverage	# PSM	# Peptides
Myofilaments and cytoskeleton											
979	P10916	MYL2	Myosin regulatory light chain 2	0.01		1.97	2.17	928.22	74.10	10	32
1820	P14649	MYL6B	Myosin light chain 6B	0.00	1.48		1.49	707.71	46.15	12	26
1851	P14649	MYL6B	Myosin light chain 6B	0.00	1.57		1.92	543.48	51.44	12	39
917	P14649	MYL6B	Myosin light chain 6B	0.01	1.20		1.32	258.04	26.44	5	9
1930	Q00872	MYBPC1	Myosin-binding protein C	0.01		-1.25		2236.12	36.20	32	90
683	P68133	ACTA1	Actin, alpha skeletal muscle	0.00		1.29	1.14	273.70	16.71	5	9
714	P68133	ACTA1	Actin, alpha skeletal muscle	0.01	-1.24	1.19		395.01	22.02	6	12
1825	P13805	TNNT1	Troponin T	0.00	1.52		1.43	1922.85	31.65	8	60
1826	P13805	TNNT1	Troponin T	0.00	1.76		1.36	349.24	32.01	8	14
1859	P13805	TNNT1	Troponin T	0.00	1.17		1.18	1756.11	32.37	11	92
1870	P13805	TNNT1	Troponin T	0.02	1.27		1.37	786.61	31.65	9	41
1873	P13805	TNNT1	Troponin T	0.02	1.15		1.19	1500.89	32.01	10	65
1869	P13804	TNNT1	Troponin T	0.00	1.31		1.35	343.77	32.01	8	19
754	P45378	TNNT3	Troponin T	0.00	-1.24		-1.22	807.77	33.09	9	28
1896	P45378	TNNT3	Troponin T	0.01		-1.31	-1.36	714.60	35.32	11	38
1926	P45378	TNNT3	Troponin T	0.01	-1.45			1218.64	36.06	11	82
793	P47755	CAPZA2	F-actin-capping protein subunit alpha-2	0.01			1.29	743.90	38.11	7	22
962	P02585	TNNC2	Troponin C	0.00		1.29	1.48	138.96	25.62	3	5
988	Q9Y281	CFL2	Cofilin-2	0.00		1.20	1.16	1196.75	46.99	7	32
745	Q9GZV1	ANKRD2	Ankyrin repeat domain-containing protein 2	0.04	1.26			883.99	38.89	12	29
Signal transduction											
1844	Q9NP98	MYOZ1	Myozenin-1	0.00	-1.28	1.40		275.58	25.42	4	6
1901	P08758	ANXA5	Annexin A5	0.04	-1.30			887.66	36.88	11	38
Energy metabolism											
1941	P06732	CKM	Creatine kinase M-type	0.00	1.17		1.25	2823.19	50.13	17	132
330	P11217	PYGM	Glycogen phosphorylase	0.02	-1.37			2682.99	49.76	35	116
340	P11217	PYGM	Glycogen phosphorylase	0.01		-1.58	-1.81	1086.55	24.11	17	36
341	P11217	PYGM	Glycogen phosphorylase	0.00	-1.42		-1.55	1417.70	32.19	24	47
347	P11217	PYGM	Glycogen phosphorylase	0.03			-1.58	514.71	11.40	8	15
350	P11217	PYGM	Glycogen phosphorylase	0.01	-1.28		-1.28	1884.55	39.07	28	62
353	P11217	PYGM	Glycogen phosphorylase	0.00	-1.27		-1.34	3084.83	46.56	36	114
355	P11217	PYGM	Glycogen phosphorylase	0.01	-1.42		-1.36	1328.94	37.17	26	47
			NADH shuttle								
735	P17174	GOT1	Aspartate aminotransferase	0.05		-1.20	-1.13	839.49	38.98	13	32
			Glycolysis								
234	P60174	TPI1	Triosephosphate isomerase	0.03	1.32			159.31	15.03	3	6
963	P60174	TPI1	Triosephosphate isomerase	0.00	1.36		1.42	2740.04	34.27	9	95
1841	P60174	TPI1	Triosephosphate isomerase	0.00		-1.22	-1.36	317.14	21.33	5	10

1842	P60174	TPI1	Triosephosphate isomerase	0.00	-1.30		-1.47	2676.12	58.39	14	76
1845	P60174	TPI1	Triosephosphate isomerase	0.00	-1.17	-1.30	-1.51	1827.49	66.08	14	83
1846	P60174	TPI1	Triosephosphate isomerase	0.01			-1.28	1011.75	59.44	12	48
1876	P60174	TPI1	Triosephosphate isomerase	0.01	-1.27		-1.15	2021.37	65.73	13	89
1879	P60174	TPI1	Triosephosphate isomerase	0.01	-1.23		-1.33	3832.78	76.92	18	177
1884	P60174	TPI1	Triosephosphate isomerase	0.00	1.30		1.31	1131.08	38.81	7	55
1862	P60174	TPI1	Triosephosphate isomerase	0.03	-1.18		-1.15	1542.68	65.73	13	63
773	P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.02	-1.18		-1.13	1562.75	42.39	11	42
658	P06733	ENO1	Alpha-enolase	0.01			-1.27	1350.74	46.31	14	38
672	P13929	ENO3	Beta-enolase	0.00		-1.28	-1.37	1356.30	39.40	13	37
677	P13929	ENO3	Beta-enolase	0.00	-1.16		-1.35	1862.95	40.09	13	53
748	P13929	ENO3	Beta-enolase	0.00	-1.24		-1.23	2358.09	25.81	9	65
1857	P13929	ENO3	Beta-enolase	0.00	-1.40			1603.79	37.56	12	57
1858	P13929	ENO3	Beta-enolase	0.00		-1.21	-1.28	931.03	32.03	9	40
1874	P13929	ENO3	Beta-enolase	0.00	-1.24		-1.27	2394.99	52.07	17	85
1908	P15259	PGAM2	Phosphoglycerate mutase 2	0.02			-1.25	81.68	29.64	5	8
561	P14618	PKM	Pyruvate kinase isozymes M1/M2	0.00	-1.39		-1.71	1593.10	42.75	19	49
562	P14618	PKM	Pyruvate kinase isozymes M1/M2	0.00		-1.31	-1.43	1456.26	41.43	20	50
			Gluconeogenesis								
799	O00757	FBP2	Fructose-1,6-bisphosphatase isozyme 2	0.00	1.50	-1.32		918.61	32.74	9	26
			Lipid metabolism								
837	Q13011	ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	0.05			1.33	622.15	17.99	6	15
839	Q13011	ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	0.03	1.33		1.42	865.19	29.57	8	22
1486	P05413	FABP3	Fatty acid-binding protein	0.02		1.45	1.29	737.15	63.91	9	23
1856	P16219	ACADS	Short-chain specific acyl-CoA dehydrogenase	0.03		-1.29		324.54	25.49	8	19
Detoxification, cytoprotection											
928	P04179	SOD2	Superoxide dismutase [Mn]	0.00		-1.43	-1.56	582.71	38.74	7	21
937	P32119	PRDX2	Peroxiredoxin-2	0.00	-1.30	1.20		220.48	24.24	5	8
1866	P05091	ALDH2	Aldehyde dehydrogenase	0.04	1.17			1340.34	40.43	15	48
1865	P49189	ALDH9A1	4-trimethylaminobutyraldehyde dehydrogenase	0.01		-1.34		543.94	25.91	11	27
815	Q9HC38	GLOD4	Glyoxalase domain-containing protein 4	0.03	1.33		1.39	388.91	17.57	4	10
468	P38646	HSPA9	Stress-70 protein	0.05	1.16			1131.14	34.02	16	33
496	P08107	HSPA1A	Heat shock 70 kDa protein 1A/1B	0.02	1.12		1.20	1018.07	28.55	16	33
893	P04792	HSPB1	Heat shock protein beta-1	0.01		1.26	1.25	515.69	45.85	7	17
960	O14558	HSPB6	Heat shock protein beta-6	0.00	1.21		1.32	763.42	45.00	6	31
958	P02511	CRYAB	Alpha-crystallin B chain	0.00	1.13		1.23	1500.55	62.86	11	76
619	P78371	CCT2	T-complex protein 1 subunit beta	0.05	1.18			782.59	18.13	7	20
885	P07451	CA3	Carbonic anhydrase 3	0.00	1.46		1.62	753.80	30.77	6	23
887	P07451	CA3	Carbonic anhydrase 3	0.02	1.22		1.29	754.90	45.77	8	26
1821	P07451	CA3	Carbonic anhydrase 3	0.00	1.23	1.18	1.45	911.33	45.38	8	28
1822	P07451	CA3	Carbonic anhydrase 3	0.00	1.41	1.17	1.66	1025.77	43.85	8	31
Proteolysis											

563	P54578	USP14	Ubiquitin carboxyl-terminal hydrolase 14	0.00	-1.25	1.20		742.77	23.08	9	18
850	P25786	PSMA1	Proteasome subunit alpha type1	0.01		1.13	1.23	429.67	36.50	7	15
1854	P28070	PSMB4	Proteasome subunit beta type-4	0.03		1.17	1.14	508.68	32.95	5	12
Membrane repair											
620	Q6ZMU5	TRIM72	Tripartite motif-containing protein 72	0.00	1.43		1.33	414.51	23.69	8	12
1885	Q6NZI2	PTRF	Polymerase I and transcript release factor	0.03	1.20			432.22	27.44	8	14
Miscealenous											
2101	P68871	HBB	Hemoglobin subunit beta	0.01		-1.48	-1.41	402.85	49.66	6	21

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- **FIGURE LEGENDS**

Figure 1. Representative 2DGE image obtained from total protein extracts of human vastus lateralis skeletal muscle. 2DGE was performed using a pH range of 5-8. Protein loading was 700 µg, and the gel was stained using colloidal Coomassie blue G-250. Differentially expressed and identified proteins are marked and spot numbers refer to Table 1.

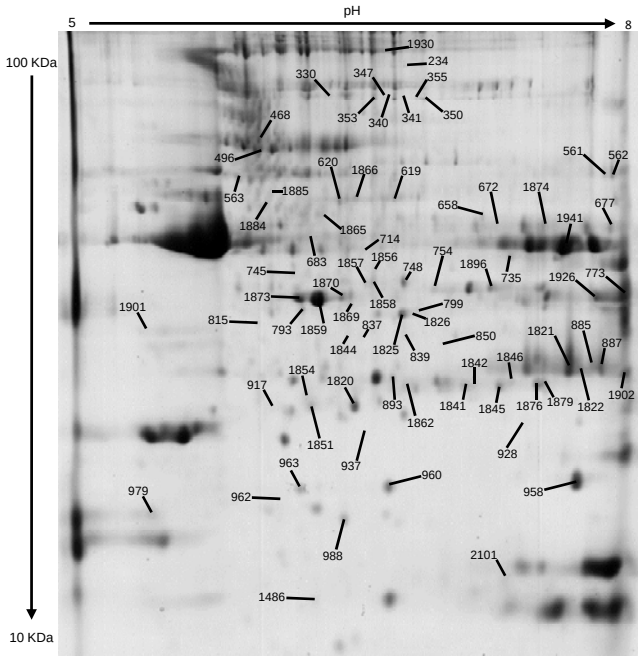
Figure 2. Example of differential expression of proteins associated to myofibrillar filaments. Representative sections of 2DGE images and representative Western blot for CAPZA2 (A) and ANKRD2 (B). In each panel, histograms represent Western blot quantification (n=7) for young (YO), healthy old men (EL) and old men with metabolic syndrome (EL-MS). Results are indicated as mean ± SE. * $p < 0.05$ indicates significant difference between YO or EL individuals.

Figure 3. Example of differential expression of proteins implicated in signal transduction. Representative sections of 2DGE images and representative Western blot for Myozenin-1. In each panel, histograms represent Western blot quantification (n=7) for healthy old men (EL) and old men with metabolic syndrome (EL-MS). Results are indicated as mean ± SE. * $p < 0.05$ indicates significant difference with EL individuals.

Figure 4. Example of differential expression of proteins implicated in energy metabolism. Representative sections of 2DGE images and representative Western blot for GAPDH (A), ACADS (B) and ECH1 (C). In each panel, histograms represent Western blot quantification (n=7) for young (YO), healthy old men (EL) and old men with metabolic syndrome (EL-MS). Results are indicated as mean ± SE. * $p < 0.05$ indicates significant difference between YO or EL individuals.

Figure 5. Example of differential expression of proteins implicated in cytoprotection and cytodetoxification. Representative sections of 2DGE images and representative Western blot for SOD2 (A), ALDH2 (B), CRYAB (C), CCT2 (D) and CA3 (E). In each panel, histograms represent Western blot quantification (n=7) for young (YO), healthy old men (EL) and old men with metabolic syndrome (EL-MS). Results are indicated as mean ± SE. * $p < 0.05$ indicates significant difference between YO or EL individuals.

Figure 6. Example of differential expression of proteins implicated in proteolysis. Representative sections of 2DGE images and representative Western blot for PSMA1. In each panel, histograms represent Western blot quantification (n=7) for healthy old men (EL) and old men with metabolic syndrome (EL-MS). Results are indicated as mean ± SE. * $p < 0.05$ indicates significant difference with EL individuals.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745
748
1896
735
1926
773
1870
1873
1858
799
1901
815
1869
1826
793
1859
837
850
1821
885
1844
1825
839
887
1842
1846
1854
1820
917
1902
893
1841
1845
1876
1879
1822
1862
1851
928
963
937
960
979
962
958
988
2101
1486

Figure 1

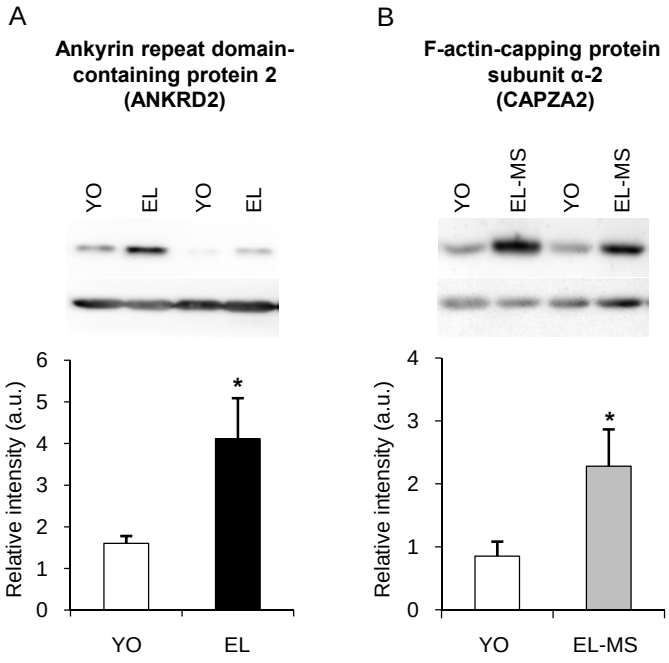
A
Ankyrin repeat domain-containing protein 2 (ANKRD2)
YO
EL
YO
EL
Relative intensity (a.u.)
0
1
2
3
4
5
6
*
YO
EL
B
F-actin-capping protein subunit α-2 (CAPZA2)
YO
EL-MS
YO
EL-MS
Relative intensity (a.u.)
0
1
2
3
4
*
YO
EL-MS

Figure 2

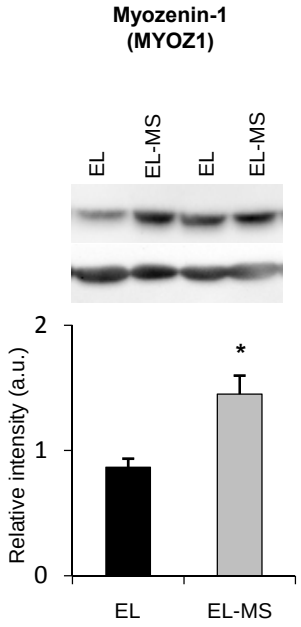
Myozenin-1
(MYOZ1)
EL
EL-MS
EL
EL-MS
Relative intensity (a.u.)
2
1
0
*
EL
EL-MS

Figure 3

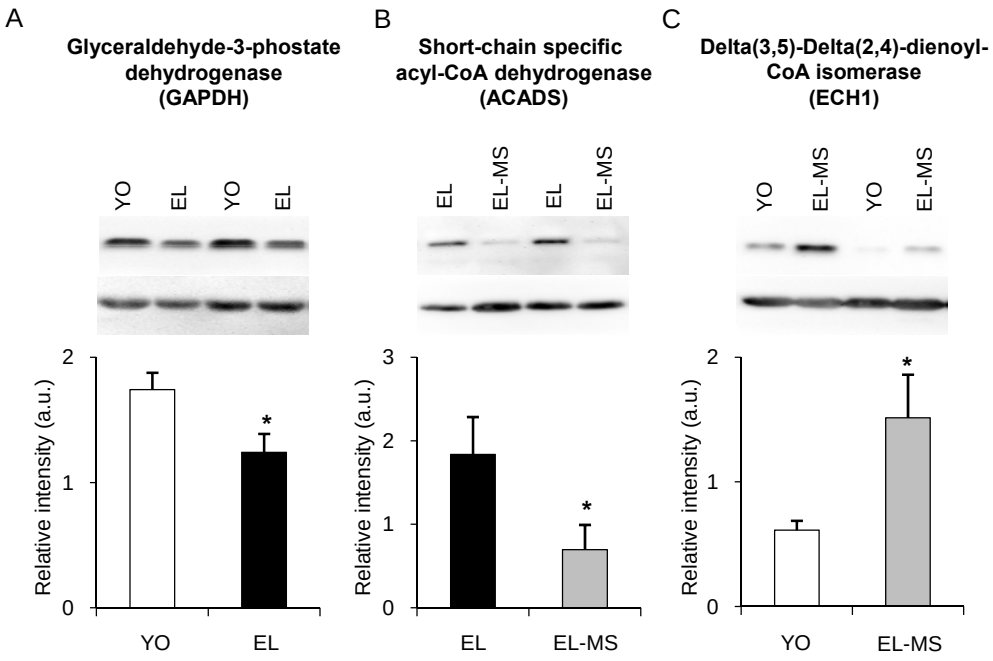
A
Glyceraldehyde-3-phostate dehydrogenase (GAPDH)
YO
EL
YO
EL
Relative intensity (a.u.)
2
1
0
*
YO
EL
B
Short-chain specific acyl-CoA dehydrogenase (ACADS)
EL
EL-MS
EL
EL-MS
Relative intensity (a.u.)
3
2
1
0
*
EL
EL-MS
C
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase (ECH1)
YO
EL-MS
YO
EL-MS
Relative intensity (a.u.)
2
1
0
*
YO
EL-MS

Figure 4

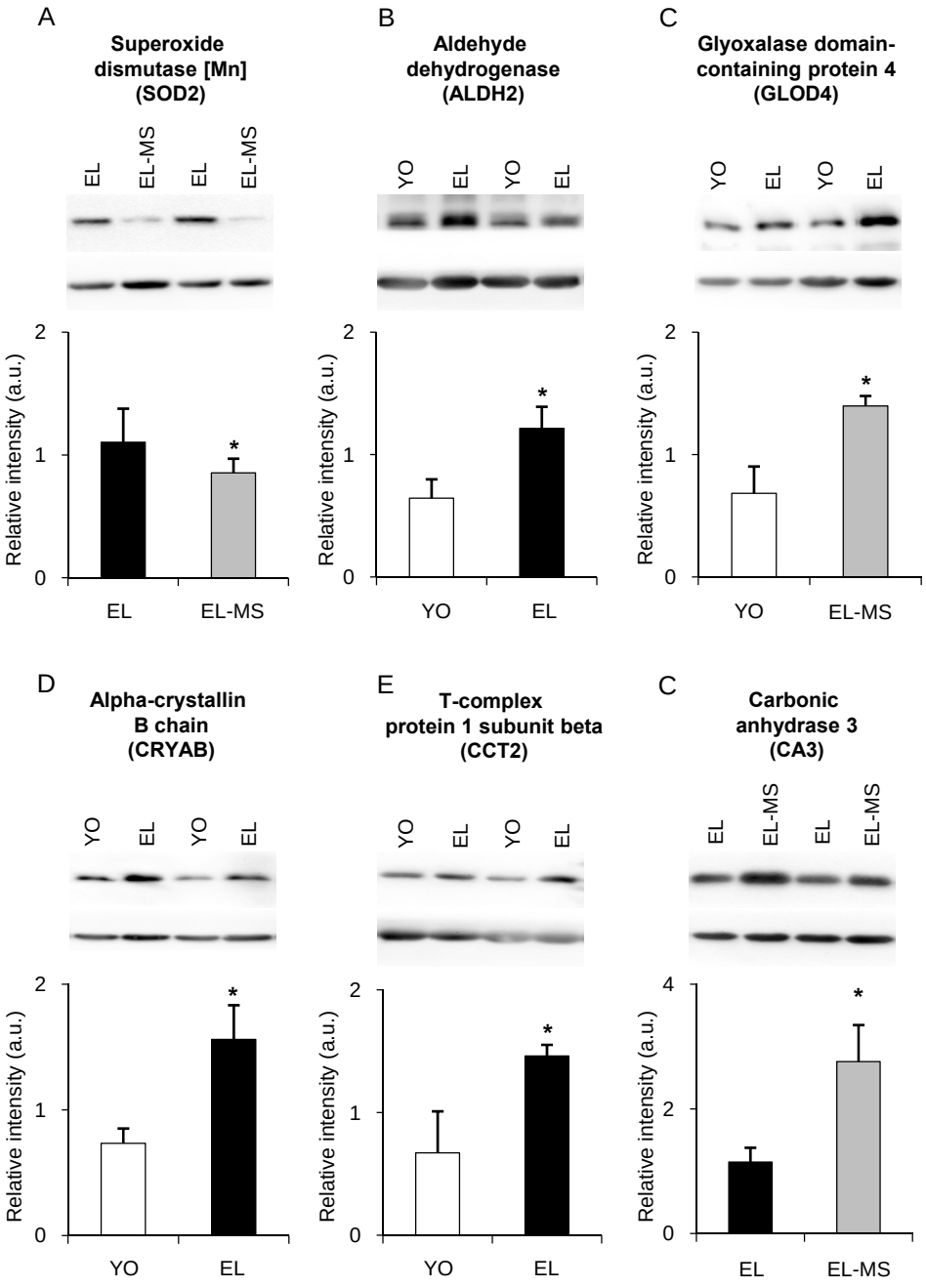
A
Superoxide dismutase [Mn] (SOD2)
EL
EL-MS
EL
EL-MS
Relative intensity (a.u.)
2
1
0
*
EL
EL-MS
B
Aldehyde dehydrogenase (ALDH2)
YO
EL
YO
EL
Relative intensity (a.u.)
2
1
0
*
YO
EL
C
Glyoxalase domain-containing protein 4 (GLOD4)
YO
EL
YO
EL
Relative intensity (a.u.)
2
1
0
*
YO
EL-MS
D
Alpha-crystallin B chain (CRYAB)
YO
EL
YO
EL
Relative intensity (a.u.)
2
1
0
*
YO
EL
E
T-complex protein 1 subunit beta (CCT2)
YO
EL
YO
EL
Relative intensity (a.u.)
2
1
0
*
YO
EL
C
Carbonic anhydrase 3 (CA3)
EL
EL-MS
EL
EL-MS
Relative intensity (a.u.)
4
2
0
*
EL
EL-MS

Figure 5

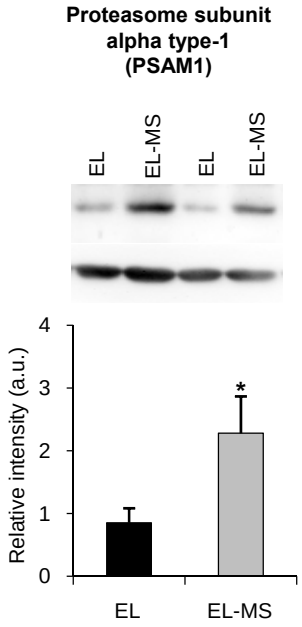
Proteasome subunit
alpha type-1
(PSAM1)
EL
EL-MS
EL
EL-MS
Relative intensity (a.u.)
0
1
2
3
4
*
EL
EL-MS

Figure 6

Discussion générale

Le vieillissement musculaire, aussi appelé sarcopénie, est un phénomène complexe et multifactoriel dont les mécanismes moléculaires restent encore mal connus. De plus, le vieillissement est également un facteur connu pour favoriser l'apparition de certaines pathologies, comme le syndrome métabolique (SM). Ce syndrome est un ensemble de facteurs de risque pour les maladies cardiovasculaire et le diabète de type II. Malgré sa forte prévalence chez les personnes âgées au niveau mondial, peu d'études se sont intéressées à l'impact du syndrome métabolique sur le muscle squelettique vieillissant.

Les objectifs de cette thèse étaient de (1) identifier de nouveaux marqueurs du vieillissement musculaire chez l'homme et chez la femme post-ménopausée, et (2) de mettre en évidence les altérations du muscle *vastus lateralis* chez l'homme âgé sain ou atteint de syndrome métabolique. Les données obtenues au cours de cette thèse nous ont permis de répondre à ces objectifs et d'apporter de nouvelles connaissances quant au vieillissement musculaire. L'ensemble de ces résultats seront discutés dans ce chapitre.

I. Le vieillissement musculaire chez l'homme et chez la femme post-ménopausée

➢ Altérations de l'appareil contractile : de la fibre musculaire aux myofilaments

Depuis le début des années 1980, de nombreuses études se sont intéressées aux altérations du muscle squelettique chez les personnes âgées, et il a été montré que le vieillissement était associé à une diminution de l'aire (atrophie) et du nombre de fibres musculaires (Lexell et al, 1983 ; Lexell et al, 1988). Par la suite, plusieurs études ont montré que cette atrophie affecte préférentiellement les fibres de type II tandis que l'aire des fibres de type I montrent peu de changements lors du vieillissement. Notre analyse

d'immunohistologie, réalisée à partir de coupes transversales du muscle *vastus lateralis*, a permis de confirmer la diminution de l'aire des fibres de type IIA et de type IIX, ainsi que des fibres hybrides I-IIA et IIA-IIX. Bien que les conséquences de cette atrophie spécifique des fibres de type II reste encore à approfondir, de récentes données indiquent la perte de masse musculaire squelettique semble être attribuée à la réduction de la taille de ces fibres (Nilwik et al, 2013). Ainsi, il est probable que la perte de masse musculaire que nous observons chez les personnes âgées pourrait en partie être la conséquence de l'atrophie de ces fibres de type II.

Contrairement à l'aire, les changements liés à l'âge de la proportion des différents types de fibres musculaires, dans le muscle *vastus lateralis*, sont très controversés dans la littérature. En effet, alors que certains auteurs n'observent pas ou peu de changement dans la proportion des différents types de fibres (D'Antona et al, 2003 ; Klitgaard et al, 1990 ; Kosek et al, 2006; Lexell & Taylor, 1991 ; Lexell et al, 1988), d'autres montrent une augmentation significative des fibres de type I (Dreyer et al, 2006 ; Poggi et al, 1987 ; Verdijk et al, 2007), ou une diminution significative des fibres de type II (Dreyer et al, 2006 ; Nilwik et al, 2013; Verdijk et al, 2007). Notre analyse d'immunohistologie n'a montré aucune différence significative dans la proportion des différents types. Cependant, suite aux résultats obtenus lors d'une étude protéomique réalisée à partir de biopsies du muscle vastus lateralis de personnes jeunes et âgées, Gelfi *et al.* suggère que le vieillissement est associé à un changement d'expression des différentes isoformes de MHCs conduisant une transition d'un phénotype musculaire rapide vers un phénotype musculaire lent (Gelfi et al, 2006). Chez l'homme, les résultats de notre étude protéomique semblent confirmer cette transition. En effet, nous avons observé une augmentation de l'expression de protéines myofibrillaires associées à un phénotype lent, comme MYL6B (Myosin Light Chain 6B), TNNT1 (slow troponin T) ou ANKRD2 (Ankyrin repeat domain-containing protein 2), et une diminution de l'expression de plusieurs isoformes de la troponine T associée à un phénotype rapide (TNNT3) dans le muscle âgé. Ainsi, bien qu'aucun changement significatif n'ait été observé dans la proportion de différents types de fibres musculaires lors du vieillissement, nos résultats montrent qu'une transition vers un phénotype plus lent semble se mettre en place. Chez la femme âgée post-ménopausée, nous n'avons pas observé cette transition, mais une expression différentielle de protéines associées au cytosquelette, tels que la vinculine et FHL3 (Four and Half LIM domain protein 3) est notée. Ces deux protéines sont localisées au niveau du disque Z et une altération de leur expression peut conduire à une désorganisation des sarcomères et donc à une perte de l'intégrité des fibres musculaires.

Dans leur ensemble, ces modifications peuvent résulter dans la perte de force musculaire et en une vitesse réduite de contraction, et contribuer ainsi au développement de la sarcopénie.

- **Perturbations du métabolisme énergétique**

Dans le muscle squelettique, le glucose est un substrat majeur et essentiel pour la production d'énergie *via* la glycolyse, nécessaire à la contraction musculaire. Dans des muscles de rats, plusieurs études protéomiques ont montré une diminution de l'expression de plusieurs enzymes glycolytiques (Triosephosphate isomérase, énolase) (Capitanio et al, 2009; Piec et al, 2005), et cette diminution a également été retrouvée chez l'homme (Gelfi et al, 2006). Nos analyses du protéome musculaire chez l'homme et la femme âgés confirment cette baisse, ce qui suggère que le vieillissement est fortement associé à une altération du métabolisme glycolytique et donc, de la glycolyse. A l'inverse, les résultats obtenus sur l'expression des enzymes mitochondriales intervenant dans le cycle de Krebs et dans les phosphorylations oxydatives sont beaucoup plus controversés. Alors que certains montrent une augmentation significative (Gelfi et al, 2006), d'autres ont mis en évidence une diminution de l'expression de ces enzymes oxydatives lors du vieillissement (Lombardi et al, 2009; Piec et al, 2005 ; Short et al, 2005). Chez l'homme, notre analyse protéomique n'a montré aucun changement au niveau des enzymes mitochondriales. Ces résultats ont été confirmés par un marquage histologique de l'activité de la cytochrome c oxydase, enzyme mitochondriale impliquée dans la phosphorylation oxydative, où aucun différence significative entre les personnes âgées et les personnes jeunes n'a été observée dans les fibres musculaires les plus oxydatives (type I, I-IIA et IIA). Cependant, l'analyse protéomique chez la femme montre une diminution de l'expression de plusieurs enzymes, comme deux sous-unités de la pyruvate déshydrogénase, l'aconitate hydratase, la furamate hydratase ainsi que des composants des complexes I, III IV et V de la chaîne respiratoire.

Plusieurs auteurs ont rapporté la présence d'un dysfonctionnement de la fonction mitochondriale lors du vieillissement (Peterson et al, 2012), et des mutations de l'ADN mitochondrial (Chabi et al, 2005), une baisse de la production de l'ATP (Short et al, 2005) ou encore une altération de la biogénèse mitochondriale (Crane et al, 2010) ont été décrits dans la littérature. Cependant, la plupart des altérations de la fonction mitochondriale liées au

vieillissement semblent, en réalité, le résultat de l'inactivité physique. En effet, plusieurs études n'ont montré aucun changement de l'activité enzymatique mitochondriale, de la respiration ou du flux d'ATP lorsque les personnes âgées ont le même niveau d'activité physique que les personnes jeunes avec lesquelles elles ont été comparées (Lanza et al, 2008 ; Larsen et al, 2012; Safdar et al, 2010). Etant donné que les hommes jeunes et âgés composant notre cohorte ont un niveau d'activité identique, ceci pourrait alors expliquer nos résultats concernant la capacité oxydative chez l'homme âgé. En ce qui concerne les femmes post-ménopausées, les données concernant leur niveau d'activité sont moins précises. Cependant, les femmes post-ménopausées âgées présentent effectivement un niveau d'activité plus faible que les femmes post-ménopausées matures, ce qui pourrait expliquer la diminution de l'expression de nombreuses enzymes mitochondriales chez ces dames âgées.

En plus du glucose, les lipides sont également une source d'énergie importante pour le muscle squelettique. Ces lipides sont stockés dans le muscle principalement sous forme de triglycérides dans des gouttelettes lipidiques, et servent de substrats à la β-oxydation mitochondriale pour produire de l'ATP. Lors du vieillissement, une accumulation élevée de gouttelettes lipidiques pourrait être associée à une réduction liée à l'âge de l'oxydation mitochondriale, conduisant à une augmentation de métabolites d'acides gras intracellulaires. Ces derniers peuvent être la cause de perturbations métaboliques comme l'apparition de la résistance à l'insuline. Comme l'indiquent notre analyse histologique, les hommes âgés sains de notre cohorte montrent une légère augmentation de l'accumulation lipidique intramyocellulaire. De plus, chez l'homme, notre étude protéomique a permis d'identifier une augmentation de l'expression d'une protéine intervenant dans la β-oxydation, la delta(3,5)-delta(2,4)-dienoyl-CoA isomérase (ECH1). Ceci suggère que chez l'homme âgé actif dont l'activité mitochondriale est peu altérée, le métabolisme lipidique ne semble pas affecté par le vieillissement. Chez la femme âgée post-ménopausée, l'analyse protéomique a révélé par contre une perturbation de l'expression de deux protéines liées aux acides gras : FABP3 et FABP4. FABP3 permet l'absorption des acides gras par le muscle squelettique et son expression diminue chez la femme âgée. FABP4 est un marqueur d'adipocyte, mais cette protéine est également connue comme étant un régulateur du stress du réticulum endoplasmique (RE) induit par des lipides toxiques. Son expression est augmentée chez la femme âgée post-ménopausée, suggérant une possible augmentation du stress-RE lors du vieillissement.

En résumé, l'ensemble de ces résultats permettent de confirmer la diminution de l'activité glycolytique dans le muscle squelettique lors du vieillissement chez l'homme mais aussi chez la femme. Cependant, alors que le métabolisme oxydatif semble peu affecté chez l'homme actif, de nombreuses enzymes mitochondriales intervenant dans le cycle de Krebs ou dans la phosphorylation oxydative ont une expression diminuée chez la femme âgée peu active. Ceci pourrait suggérer que l'activité physique permettrait de prévenir des altérations au niveau de la capacité mitochondriale et ainsi de fournir assez d'énergie pour le bon fonctionnement musculaire. Enfin, lors du vieillissement, nos résultats n'ont pas montré de perturbations majeures du métabolisme lipidique.

- **Mise en place de mécanismes de cytodétoxification et cytoprotection dans le muscle squelettique âgé**

Protection contre le stress oxydatif

Les espèces réactives de l'oxygène, aussi appelées ROS (Reactive Oxygen Species), sont des produits dérivés du métabolisme mitochondrial pouvant causer des dommages cellulaires en oxydant les lipides, les protéines ou même les acides nucléiques. Plusieurs études ont montré que le vieillissement musculaire était associé à l'apparition d'un stress oxydatif important (Baraibar et al, 2013). Les études protéomiques différentielles du vieillissement musculaire chez l'homme et la femme nous ont permis de mettre en évidence une perturbation de l'expression de plusieurs protéines anti-oxydantes, tels que la péroxirédoxine, la catalase ou encore la protéine DJ-1, ce qui témoigne de la présence d'un stress oxydatif dans le muscle âgé.

Détoxification de produits cytotoxiques

Le stress oxydatif conduit à la production d'aldéhydes cytotoxiques, pouvant réagir avec les protéines cellulaires, les acides nucléiques ainsi qu'avec les membranes cellulaires. Précédemment, des études protéomiques du vieillissement musculaire chez le rat ont mis en évidence une augmentation d'enzymes de détoxification, tels que l'aldéhyde déshydrogénase (ALDH2), permettant l'élimination et la protection contre ces produits cytotoxiques (Lombardi et al, 2009; Piec et al, 2005). Nos résultats, issus de l'analyse du protéome

musculaire chez l'homme et la femme post-ménopausée âgés, confirment la surexpression d'ALDH2 et mettent également en évidence la surexpression d'autres enzymes de détoxification tels que l'alcool déshydrogénase et une protéine appartenant au système glyoxalase (GLOD4, Glyoxalase domain-containing protein 4). Ainsi, la surexpression de ces protéines semble suggérer une augmentation de la prise en charge et de l'élimination des aldéhydes cytotoxiques lors du vieillissement.

Contrôle qualité des protéines cellulaires

La détection, le repliement et éventuellement l'élimination des protéines anormales est permise par des protéines chaperonnes, dont la plupart sont des protéines de choc thermique appelées HSP (Heat Shock Protein) ainsi que des chaperonines. Une observation majeure de nos travaux est la surexpression globale lors du vieillissement de 6 HSPs qui sont HSPA1A (ou HSP70), HSPA9 (ou GRP75), HSPB1 (ou HSP27), HSPB5 (ou alpha crystallin B chain), HSPB6 (ou HSP20) et HSP90-β. Bien que chacune de ces protéines ait une fonction spécifique dans la cellule, ce sont toutes des protéines essentielles pour l'homéostasie protéique (la proteostasie) et elles préviennent l'agrégation de protéines mal-repliées. De plus, l'augmentation de l'expression de la protéine HSPA1A permettrait de prévenir l'atrophie musculaire (Senf et al, 2008). Chez l'homme âgé, nous avons également identifié la chaporonine CCT2 (Chaperonin Containing T-complex polypeptide 1 subunit 2) qui assure le repliement des certaines protéines du cytosquelette, et en particulier de l'actine et de la tubuline, contribuant ainsi au maintien de l'intégrité myofibrillaire.

L'ensemble de ces résultats sont en accord avec l'existence d'un stress oxydatif lors du vieillissement. À notre connaissance, aucune étude protéomique portant sur le vieillissement musculaire humain n'a observé la surexpression à la fois d'enzymes anti-oxydantes, d'enzymes de détoxification mais également de protéines chaperonnes. Ceci suggère que la cellule musculaire va mettre en place un certain nombre de mécanismes de défenses afin de lutter contre le stress cellulaire qui apparait avec l'âge, ce qui contribue probablement au maintien de la fonctionnalité musculaire.

II. Altérations du muscle squelettique chez les personnes âgées atteintes de syndrome métabolique (SM)

➢ Perturbations du métabolisme énergétique associées au syndrome métabolique

Afin d'assurer une production d'ATP suffisante pour le fonctionnement musculaire, le métabolisme énergétique est dépendant des quantités de substrats disponibles. Comme nous l'avons évoqué précédemment, le glucose est le substrat majeur de la cellule musculaire pour produire de l'énergie *via* le métabolisme glycolytique suivi ou non par le métabolisme oxydatif. Comme l'indiquent notre analyse protéomique, le muscle squelettique d'hommes âgés atteints de syndrome métabolique possède une diminution de l'expression de plusieurs enzymes de la glycolyse comme l'énolase, la pyruvate kinase ou la phosphoglycérate mutase-2. De plus, certaines étapes de la glycolyse nécessitent la présence de coenzymes réduits cytosoliques, et notamment de NAD^+. Des systèmes de navette, présents au niveau de la membrane mitochondriale, vont permettre de transférer le NADH issu de la glycolyse vers la matrice mitochondriale en régénérant du NAD^+ dans le cytosol. Nos données montrent que l'expression de l'aspartate aminotransférase cytosolique (GOT1), enzyme essentielle dans le système de navette malate-aspartate, est diminuée chez les personnes âgées atteintes de SM. Ces résultats semblent ainsi traduire une diminution de la régénération du NAD^+ qui serait associée à une diminution du métabolisme glycolytique.

En ce qui concerne le métabolisme oxydatif, alors que le marquage histochimique de l'activité de la cytochrome c oxydase (COX) n'a montré aucune différence significative lors du vieillissement, nous avons observé que le syndrome métabolique est associé à une diminution importante de l'activité de cette enzyme dans les différents types de fibres musculaires. Il semble donc que la capacité oxydative soit altérée chez la personne âgée ayant un SM. Cependant, l'étude protéomique différentielle du vieillissement musculaire chez l'homme n'a pas mise en évidence un changement d'expression des enzymes mitochondriales oxydatives lors du SM. Néanmoins, bien que les analyses protéomiques intégratives constituent un outil puissant pour appréhender les réponses biologiques et leurs régulations dans leur globalité, elles ne permettent d'analyser qu'une portion réduite du protéome et le changement d'expression de certaines enzymes ne traduit en aucun cas un changement au

niveau de leur activité. En effet, certaines modifications post-traductionnelles, tels que la phosphorylation ou l'oxydation, peuvent venir modifier l'activité enzymatique de certaines protéines sans que leur expression ne soit perturbée. Ainsi, nous faisons l'hypothèse que le syndrome métabolique est associé à des altérations de l'activité de certaines enzymes mitochondriale, ce qui va réduire la capacité oxydative du muscle squelettique, sans changement majeur des niveaux protéiques de ces enzymes.

Dans le muscle squelettique, le métabolisme du glucose est stimulé par l'insuline. Le vieillissement est généralement associé à une altération de cette stimulation par l'insuline, ce qui peut aboutir à l'apparition d'une résistance à l'insuline, composant majeur du syndrome métabolique (Reaven, 1995). De plus, chez les personnes insulino-résistantes, une diminution du métabolisme oxydatif est fréquemment associé à une augmentation de l'accumulation des gouttelettes lipidiques intramyocellulaires (Goodpaster et al, 2001; van Loon & Goodpaster, 2006). Au cours de cette thèse, nous avons quantifié cette accumulation *via* un marquage à l'huile rouge, et nous avons mis en évidence une augmentation très importante de la quantité de gouttelettes dans les muscles des hommes âgés ayant un syndrome métabolique, notamment dans les fibres lentes oxydatives de type I. Notre analyse protéomique a également révélé une surexpression de FABP3, protéine de liaison des acides gras, ce qui est cohérent avec un contenu en lipides myocellulaires élevé. En parallèle, cette analyse a mis en évidence une perturbation de l'expression de deux protéines intervenant dans la β-oxidation mitochondriale des acides gras : l'acyl-CoA déshydrogénase spécifique des chaînes courtes (ACADS ; en anglais, Short-chain specific acyl-CoA dehydrogenase) et la delat(3,5)-delta(2,4)-dienoyl-CoA isomérase (ECH1). Ainsi, chez les personnes âgées atteintes de syndrome métabolique, cette accumulation élevée de gouttelettes semble associée à une réduction de l'oxydation mitochondriale. Ceci peut alors conduire à une augmentation de métabolites d'acides gras intracellulaires (diacylglycérol, fatty acyl Coenzyme A, céramides) qui peuvent perturber la signalisation de l'insuline (Lowell & Shulman, 2005 ; Petersen et al, 2003). De plus, une étude a également montré que les gouttelettes lipidiques peuvent participer à la perte de masse du muscle squelettique (Conte et al, 2013).

En conclusion, le syndrome métabolique est associé à une altération à la fois du métabolisme glycolytique, mais aussi du métabolisme oxydatif. Nos résultats soulignent également une perturbation du métabolisme lipidique et notamment de l'expression de deux protéines (ACADS et ECH1) intervenant dans la β-oxydation, qui pourraient constituer de nouvelles

pistes afin de mieux comprendre les altérations du muscle squelettique liées au syndrome métabolique lors du vieillissement.

- **Altérations du réseau de capillaire dans le muscle squelettique de personnes âgées atteintes de syndrome métabolique**

Le réseau de capillaires constitue une vaste surface d'échanges (nutriments, eau, ions minéraux, gaz respiratoires...) entre le sang et les cellules musculaires, ce qui le rend essentiel pour le fonctionnement du muscle squelettique. Alors que nous n'avons pas observé de changement de ce réseau lors du vieillissement sain, de fortes modifications ont été mises en évidence dans le muscle squelettique des hommes âgés atteints de syndrome métabolique. Parmi ces changements, une diminution de la densité capillaire (CD), ainsi que du nombre de capillaires par fibre musculaire (C/F) ont été observés. Nous avons également montré que le syndrome métabolique chez l'homme âgé est associé à une diminution de la tortuosité (CapTor) et de la capacité d'échanges entre les capillaires et les fibres musculaires (CFPE et LC/PF).

Les conséquences de ces changements au niveau musculaire sont diverses, et une corrélation entre la capacité oxydative et le contenu en capillaire a notamment été démontrée (Charifi et al, 2004; Poole & Mathieu-Costello, 1996 ; Sullivan & Pfefferbaum, 2006). Au cours de cette thèse, une forte corrélation a été observée entre l'indice LC/PF, le nombre de capillaires par fibre et l'activité de la cytochrome c oxydase. Ainsi, la diminution de la capillarisation du muscle squelettique, associée au SM, contribue probablement à la réduction de la capacité oxydative observée précédemment. De plus, en accord avec ce qui a été décrit dans la littérature, la densité capillaire, le nombre de capillaires par fibre ainsi que les indices CFPE et LC/PF sont corrélés avec la consommation maximale d'oxygène (VO_{2max}). Ceci suggère donc qu'une perturbation du réseau capillaire pourrait contribuer à une diminution des performances physiques.

- **L'hypertension, un composant majeur du syndrome métabolique**

Parmi tous les composants du syndrome métabolique, l'hypertension est le facteur le plus prévalent chez la personne âgée (Ford et al, 2010). En étudiant les caractéristiques physiologiques de chacune des personnes âgées contrôles de notre cohorte, nous avons constaté que 60 % de ces individus présentaient en réalité une hypertension artérielle. Nous nous sommes alors demandés si cette hypertension pouvait engendrer à elle-seule des modifications au niveau du muscle squelettique lors du vieillissement. Dans la littérature, l'effet de l'hypertension sur la vascularisation, et notamment au niveau des artérioles, est assez connu. En effet, des modifications de la fonction des artérioles ont été mises en évidence au cours de l'hypertension artérielle (Feihl et al, 2006). Pour l'essentiel, il s'agit d'une hyperréactivité aux stimuli vasoconstricteurs entraînant une réduction anormale de lumières vasculaires (Hernandez et al, 1999) et d'une dysfonction endothéliale (Cardillo et al, 2002; Panza et al, 1995) en relation avec une activité réduite d'oxyde nitrique (Hansen et al, 2011; Nakamura & Prewitt, 1991). De plus, une raréfaction de capillaire a également été rapportée lors d'une hypertension dans différents organes, et notamment dans le muscle squelettique (Hansen et al, 2010; Hedman et al, 2000 ; Henrich et al, 1988). Cependant, les altérations du muscle squelettique liées à l'hypertension lors du vieillissement sont peu connues.

Dans le muscle *vastus lateralis* des hommes âgés sans syndrome métabolique appartenant à notre cohorte, nous avons constaté que l'hypertension est majoritairement associée à une perturbation de la fonction vasculaire, confirmant les données précédemment décrites dans la littérature. De plus, chez ces individus, une diminution de l'activité enzymatique de la COX ainsi qu'une augmentation de l'aire de la matrice extracellulaire ont été observées. Cependant, en comparant les personnes âgées hypertendues avec ou sans syndrome métabolique, nous n'avons observé aucune différence significative quand au contenu en capillaire, à l'activité COX ou encore à l'aire de la matrice extracellulaire. Ceci suggère alors que l'hypertension semble être majoritairement responsable des altérations vasculaires ainsi que de la diminution de la capacité oxydative du muscle squelettique associées au syndrome métabolique lors du vieillissement. Ainsi, mieux comprendre les mécanismes moléculaires responsables de l'apparition de l'hypertension chez la personne âgée permettrait de prévenir le développement d'autres pathologies comme le syndrome métabolique, ce qui contribuerait à améliorer la qualité de vie des personnes âgées.

Conclusion et perspectives

Conclusion et perspectives

Les résultats obtenus au cours de cette thèse montrent les nombreux changements au niveau du muscle squelettique qui apparaissent avec l'âge avancé. Les analyses protéomiques réalisées ont permis d'identifier de nombreux marqueurs associés au vieillissement musculaire, qui sont principalement impliqués dans l'organisation des myofilaments et du cytosquelette, dans le métabolisme énergétique, la cytodétoxification/cytoprotection, la réparation membranaire et la protéolyse. Plusieurs de ces fonctions sont également altérées chez les personnes âgées atteintes de syndrome métabolique (SM), et une perturbation de la β-oxydation des acides gras semble aussi présente. En parallèle, les analyses d'immunohistologie ont révélé une atrophie préférentielle des fibres de type II avec une déformation accrue de celles-ci lors du vieillissement. Une réduction de l'activité oxydative d'une enzyme mitochondriale (COX), une accumulation élevée de lipides intramyocellulaires ainsi qu'une forte altération du réseau de capillaires ont également été observées dans le muscle squelettique de personnes âgées atteintes de syndrome métabolique. Nous avons également rapporté un rôle prépondérant de l'hypertension quant aux altérations vasculaires associées au SM. En conclusion, nous faisons l'hypothèse que lors du vieillissement, l'altération du muscle squelettique associée à d'autres modifications physiologiques comme l'hypertension ou le cholestérol, favorise l'apparition du syndrome métabolique. Par la suite, ce syndrome va à son tour altérer le muscle squelettique, induisant une perte de masse et de force musculaire et augmentant ainsi fortement la dépendance, l'apparition de maladies cardiovasculaires et donc le taux de mortalité des personnes âgées.

Afin d'approfondir l'ensemble de résultats obtenus au cours de cette thèse, plusieurs perspectives sont envisagées. A court terme, l'étude des modifications post-traductionnelles des protéines nous paraît être indispensable, vu leur importance dans le processus de vieillissement. En effet, de nombreuses protéines dont l'expression est modifiée au cours du vieillissement ont pu être mises en évidence dans ce travail. Cependant, il est connu que certaines modifications post-traductionnelles tels que la phosphorylation ou encore l'oxydation peuvent perturber l'activité de certaines protéines sans aucun changement de leur

expression. Ainsi, la mise en évidence de ces modifications via la technique d'électrophorèse bidimensionnelle permettrait apporter un niveau d'informations supplémentaires aux analyses protéomiques. Afin de compléter nos connaissances sur le vieillissement musculaire associé ou non au syndrome métabolique, nous avons également entrepris une analyse du transcriptome à partir des biopsies musculaires de nos 39 individus à l'aide de puces Affymetrix. Les résultats, en cours d'analyse, révèlent que les profils transcriptomiques corrèlent assez bien avec les résultats protéomiques. Une autre perspective à court terme serait d'identifier la nature des lipides intramusculaires chez les personnes jeunes ou âgées avec ou sans SM. En effet, notre analyse d'imagerie moléculaire nous a permis de mettre en évidence un profil lipidique différent entre ces trois groupes. Or, du fait de la grande diversité des acides gras ainsi que le manque de bases de données, l'identification de la nature des lipides différant lors du vieillissement ou lors du SM n'a pu être réalisée. Une optimisation de la technique, passant notamment par l'utilisation des lipides standards, nous permettrait sans doute d'obtenir une meilleure identification.

Enfin, à long terme, la perspective la plus pertinente de ce travail serait de réaliser un suivi longitudinal des personnes âgées étudiées au cours de cette thèse, afin de suivre l'évolution des altérations du muscle squelettique liées au vieillissement au cours du temps. De plus, il serait également intéressant de comparer les modifications du muscle squelettique liées au vieillissement entre des personnes âgées ayant subit une fonte musculaire importante et des personnes âgées dont la masse musculaire est peu affectée. La finalité de l'ensemble de ces études sera de mieux comprendre les mécanismes de la sarcopénie et, si possible, de prévenir, ou du moins de retarder, l'échéance d'une fonte musculaire handicapante pour les personnes âgées.

Annexes

Publication 6

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"Expression and modification proteomics during skeletal muscle ageing"

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Expression and modification proteomics during skeletal muscle ageing

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Abstract Skeletal muscle ageing is characterized by a progressive and dramatic loss of muscle mass and strength leading to decreased muscular function resulting in muscle weakness which is often referred to as sarcopenia. Following the standardisation of "omics" approaches to study the genome (genomics) and the transcriptome (transcriptomics), the study of the proteins encoded by the genome, referred to as proteomics, is a tremendous challenge. Unlike the genome, the proteome varies in response to many physiological or pathological factors. In addition, the proteome is orders of magnitude more complex than the transcriptome due to post-translational modifications, protein oxidation and limited protein degradation. Proteomic studies, including the analysis of protein abundance as well as post-translational modified proteins have been shown to provide valuable information to unravel the key molecular pathways implicated in complex biological processes, such as tissue and organ ageing. In this article, we will describe proteomic approaches for the analysis of protein abundance as well as the specific protein targets for oxidative damage upon oxidative stress and/or during skeletal muscle ageing.

Keywords 2D-gel based expression proteomics · Skeletal muscle · Ageing · Oxidative stress · Protein oxidation · Oxidative proteome modifications

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Introduction

Ageing is associated with a progressive decline in muscle mass and strength, a condition known as sarcopenia (Evans 1995). Estimates of the rate of muscle loss are 5 % per decade starting in the fourth decade of life. Sarcopenia is a prevalent condition, as it can be detected in 13–24 % of adults over 60 years of age, and reaches 50 % in individuals older than 80 (Baumgartner et al. 1998). Inter-individual differences in prevalence of sarcopenia will depend not only on genetic factors, but also on food habits, activity patterns and general lifestyle. Healthy skeletal muscles are central not only for coordinated movements and postural control but also for general well being. Hence, age-related loss in skeletal muscle contractile strength increases the risk of impaired mobility, poor

balance and falls. Skeletal muscle, which is the most abundant tissue in the adult body, also plays a central role as a reserve for energy and amino acids, and is a major site of fatty acid oxidation, carbohydrate metabolism and maintenance of heat homeostasis. Hence, age-related loss of muscle mass triggers severe metabolic side effects and frailty in the elderly. Following the standardization of global technologies to study genomics and transcriptomics, the study of all proteins encoded by the genome, referred to as proteomics, is a tremendous challenge. Unlike the genome, the proteome varies in response to many physiological or pathological factors. In addition, the proteome is orders of magnitude more complex than the transcriptome due to post-translational modifications, protein oxidation and limited protein degradation. The proteomic analysis of sarcopenia is also technically challenging because of the wide concentration range and the diversity of proteins expressed in muscles.

Accumulation of damaged macromolecules, including oxidative damage to proteins, is a hallmark of cellular and organismal ageing (Levine and Stadtman 2001). This accumulation has been viewed as the combined result of increased production of reactive oxygen species (ROS) and other toxic compounds coming from both cellular metabolism and external factors as well as the failure of protein maintenance (i.e. degradation and repair) with age (Friguet 2006; Petropoulos and Friguet 2006). Protein oxidation is particularly detrimental as the resulting damage can render oxidized proteins inactive and lead to cellular functional abnormalities. Among the protein modifications that have been observed during ageing at the level of both the cells and the organ, oxidative modifications of proteins have been particularly well described. At the cellular level, an age-related accumulation of modified proteins has been associated with an impairment of proteasom function with ageing (Baraibar and Friguet 2012; Breusing and Grune 2008; Friguet 2002; Friguet et al. 2000). Skeletal muscle is composed of many proteins specialized for contraction, including contractile and structural proteins, but also enzymes whose optimum activity is required for proper muscle function. Knowledge on the extent and impact of protein oxidative modifications that occurs during ageing is sparse regarding skeletal muscle. Indeed, cellular ageing causes oxidative modifications to the proteome but the targets of these changes and their consequences have not yet been clearly identified. This is an essential step to get a complete and general view of the oxidative modification of proteins and their involvement in muscle weakness and to understand the mechanisms by which oxidized proteins accumulate and potentially affect cellular functions during cellular senescence and/or ageing in vivo. In the context of the European FP7 funded project Myoage we have applied many of the approaches described in this paper to try to increase our understanding of how modifications in the muscle proteome can influence muscle function during ageing.

Expression proteomics and muscle ageing

2D gels for proteomics of sarcopenia

The dynamic range of protein expression between the most and the least abundant proteins is not known in skeletal muscle, but was estimated in standard human cells to be as great as six orders of magnitude (Rabilloud 2002). In muscle homogenates the detection of low abundance proteins (such as signal transduction components, transcription factors) will be masked by the presence of substantial amounts of contractile proteins that represent about 55–60 % of the total muscle proteins. Strategies to enrich for low-copy number proteins are to subdivide the proteome either by isolating cell organelles, or to target a specific sub-proteome (Gorg et al. 2009). Mitochondria (O'Connell and Ohlendieck 2009) and non-ionic detergent phase extracts (Donoghue et al. 2010) have, for example, been used to investigate the proteome of the ageing skeletal muscle. Nonetheless, issues such as tissue abundance, sample preparation, cross-contamination between sub-fractions, reproducibility, sensitivity, or resolution of the analysis have influenced the choice of the techniques, and most sarcopenia studies have been based on whole muscle extracts. Two-dimensional (2D) polyacrylamide gel electrophoresis enables the separation of protein mixtures according to their p*I* in the first dimension and Mr in the second dimension. Different immobilized pH gradients (IPG) can be applied to improve 2D gel resolution, including linear and non-linear wide pH range (pH 3–10), medium (3 pH units), narrow (1 pH unit) and ultra-narrow (<1 pH unit) IPG strips casted in different pH ranges between pH 2.5 and 12 (Görg et al. 2004). The

detection and quantification of the proteins is critical as it reveals spots that exhibit statistically significant variation (Meunier et al. 2007). Ideally, detection methods should be extremely sensitive, linear over a wide concentration range, homogeneous for different proteins, and compatible with mass spectrometry (MS). Proteins are visualized either by pre-labelling (with cyanine-based dyes) the sample prior to 2D gels, or by subsequently staining (with colloidal Coomassie, silver, ruthenium or epicocconone-based dyes) the gels after separation of the proteins (Rabilloud and Lelong 2011).

A high-resolution 2D gel can simultaneously resolve 2,000–3,000 protein spots, and it is possible to quantify nearly 1 ng of protein per spot (Görg et al. 2004). It should be noted that 2D gels provide a map of mostly intact proteins that also reflects the abundance of each protein. Therefore, 2D gels are very appropriate to investigate protein isoforms, as many post translational modifications (phosphorylation, glycosylation) will change their isoelectric point and/or molecular weight, and thus shift the position of the protein in the 2D gel. Despite their high-resolution power, 2D gels nevertheless present major disadvantages such as a limited dynamic range and difficulties in resolving low abundance proteins, proteins with extreme p*I* and/or Mr, and proteins with hydrophobic properties such as membrane proteins.

Shotgun proteomics in sarcopenia research

The recognition of these limitations led to the development of alternative "shotgun" proteomics for proteome analysis. In a classical shotgun proteomic workflow, the mixture of proteins is first digested into peptides (usually with trypsin), and the peptides are then separated in an liquid chromatography (LC) column, usually by their hydrophobicity. However, different combinations of strong cation exchange, anion exchange, reverse phase, affinity chromatography, and isoelectric focusing have also been investigated (Gilmore and Washburn 2010). The solvent eluting from the LC is interfaced with a tandem mass spectrometer (MS–MS), directly through electron spray ionization (ESI) or through an offline approach using MALDI (Matrix-Assisted Laser Desorption/Ionization), leading to identification of the amino acid sequence. Because proteins are initially hydrolysed into peptides, one drawback of shotgun proteomics is that most protein isoform specific information is lost. Shotgun proteomics (1D-PAGE coupled with LC–ESI–MS/MS) has been used to characterize the proteome of human muscle whole homogenate (Hojlund et al. 2008) as well as the mitochondrial fraction (Lefort et al. 2009). This technology led to the identification of 954 and 823 different proteins, respectively. This is close to the resolution achieved using 2D gels, but importantly allowed the definition of proteins that were out of the 2D-PAGE resolution range due to high Mr, extreme p*I* or hydrophobicity.

Despite alternative/complementary approaches (shotgun proteomics, antibody arrays), 2D gels with IPGs combined with protein identification by MS is still routinely applied for quantitative expression profiling of complex protein extracts such as muscle lysates. One reason is that, in contrast to shotgun-based methods, which perform analysis on peptides, 2D gels deliver a map of intact proteins, and therefore the relative abundance of the different isoforms for each protein. Another reason is that 2D gel technology is able to perform routine parallel expression profiling of large sets (up to 24) of complex protein mixtures. Proteomic investigations of skeletal muscle ageing have led to the identification of potential sarcopenic biomarkers, involved in contractile and cytoskeletal networks, cytosolic and mitochondrial energy metabolisms, cellular stress response, detoxification mechanisms and metabolite transportation (Fig. 1).

Myofibrillar and cytoskeletal networks

Muscle movement depends on interactions between myosin-containing thick filaments and actin-containing thin filaments, and proteomic studies have

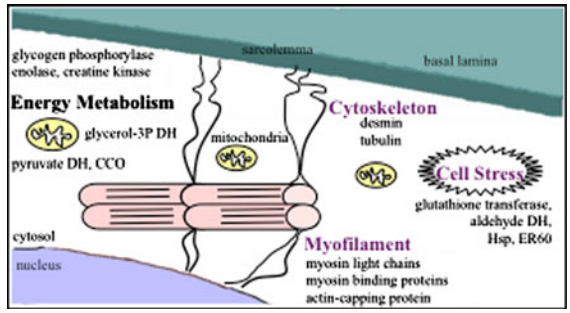

Fig. 1 Schematic diagram showing some age-related changes in the proteome of skeletal muscle. *DH* dehydrogenase, *CCO* cytochrome *c* oxidase, *Hsp* Heat shock protein

revealed that ageing is associated with perturbations of this myofibrillar network. It is important to remember that the differential expression of different isoforms of myosin heavy and light chains, will determine force and velocity in muscle. Age-dependent changes in myosin light chain isoforms have been demonstrated differentially between studies, reflecting species and/or muscle specificity. However there is a consistent age-dependent decrease in the fast isoforms of myosin light chain 1/3 and myosin regulatory light chain 2 (Gelfi et al. 2006; Capitanio et al. 2009; Gannon et al. 2009; Donoghue et al. 2010). In addition to myosin, the thick filament contains regulatory proteins that have significant effects on thickness and lateral alignment of myosin filaments. Important regulatory proteins (myosin binding proteins C and H) that control thick filament integrity are decreased during ageing (Doran et al. 2008; Capitanio et al. 2009).

Perturbation of the thin filament of the myofibrillar network have been illustrated by the differential expression of isoforms of actin and of major regulators of the thin filament, troponin and tropomyosin in rat gastrocnemius (Doran et al. 2008; Gannon et al. 2009) and human *vastus lateralis* muscle (Gelfi et al. 2006; Staunton et al. 2012). The troponin complex (troponin C, I, and T) is the sarcomeric Ca^{2+}-dependent regulator for skeletal muscle contraction. Troponin T specifically controls the position of tropomyosin near the interface between actin and myosin, and thereby mediates the activation and force development of the actomyosin contractile units (Clark et al. 2002). Different isoforms have been identified in these different studies, indicating the importance of post-translational modifications (phosphorylation) for these thin filament proteins. Muscle ageing is further associated with a reduced content of actin-capping protein CapZ (Piec et al. 2005; Gannon et al. 2009) which blocks the exchange of actin monomers and anchors the thin filament to the Z-line.

In order to compensate for this reorganization of the contractile network and to maintain the integrity of the myofibers, old muscles up-regulate cytoskeletal proteins such as desmin, tubulin and gelsolin (Piec et al. 2005; Capitanio et al. 2009; Lombardi et al. 2009). Desmin intermediate filaments constitute radial and longitudinal mechanical connections between myofibrils, and they also link sarcomeric Z-lines to the subsarcolemmal cytoskeleton (Goldfarb and Dalakas 2009). Tubulins are positioned between myofibrils and participate in the mechanical integration of various organelles in striated muscle. Therefore, while the cytoskeletal structures tend to maintain the integrity of muscle fibers during ageing, modifications in sarcomeric actomyosin and regulatory proteins may be related to some of the characteristic alterations of myofiber contractile properties in old skeletal muscles.

Energy metabolism

Disturbance in energy metabolism is another characteristic feature of old muscles. This includes the differential expression of enzymes central to cytosolic and mitochondrial bioenergetics. Several enzymes involved in anaerobic metabolism were found to be decreased in elderly muscles. Amongst these, enolase, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, and glycogen phosphorylase have been shown to decrease with age in rat (Piec et al. 2005; Capitanio et al. 2009) and human (Gelfi et al. 2006; Staunton et al. 2012) skeletal muscle. There is also an age-related decline in M-type creatine kinase, which catalyzes the transphosphorylation between phosphocreatine and ADP, phosphorylates creatine, and connects intracellular sites of energy demand with sites of ATP production.

Mitochondria are central organelles for ATP generation via oxidative phosphorylation. Changes in mitochondrial functions have long been associated with muscle ageing, and the oxidative capacity has been shown to be impaired with old age. The cumulative oxidative damage of mtDNA that occurs in the ageing muscle is associated with a reduced content of several key mitochondrial oxidative enzymes and deficiencies in the electron transport chain (Short et al. 2005). The shotgun profiling of the human proteome has identified several aerobic markers with a decreased density, including pyruvate dehydrogenase, aconitate hydratase, citrate synthase, and cytochrome *b*–*c*1 complex in old vastus lateralis mitochondria (Short et al. 2005). A similar decline in mitochondrial enzymes was found in some (Piec et al. 2005; Lombardi et al. 2009) but not all proteomic investigations (Gelfi et al. 2006; O'Connell and Ohlendieck 2009). Another important observation is the down-regulation of glycerol-3-phosphate dehydrogenase (Piec et al. 2005; Capitanio et al. 2009; Lombardi et al. 2009), which, together with its mitochondrial isoform, constitutes the glycerol-3-

phosphate dehydrogenase shuttle. Age-related decline in glycerol 3-phosphate dehydrogenase may result in a reduced mitochondrial oxidation of cytosolic NADH in old muscle.

Although detailed changes may differ according to species, obesity status (Wistar vs Lou/c/jall rats), or the age of the control group (young vs mature adults), proteomic studies agree that bioenergetics' markers partly account for the altered contractile and metabolic properties that characterize old muscles. Differences in protein isoforms that are observed in different studies may be due to changes in post-translational modifications. For example, the nitroproteome of old muscle identified enzymes (creatine kinase, aldolase, glyceraldehyde-3-P dehydrogenase) involved in energy metabolism and exhibiting increased levels of tyrosine nitration during ageing (Kanski et al. 2005). The rat muscle phosphoproteome further revealed that ageing is associated with altered phosphorylation of enzymes involved in energy metabolism, such as creatine kinase, enolase, lactate dehydrogenase, aconitase or cytochrome-*c*-oxidase (Gannon et al. 2008).

Skeletal muscles are composed of different fibre types, i.e. slow oxidative (type I), fast oxidative glycolytic (type IIA), fast glycolytic (type IIX) and hybrid fibres (Schiaffino 2010). A shift in fibre type proportion may occur during ageing, and histochemical analyses indicate that type II fibres tend to atrophy with age (Snijders et al. 2009). Proteomic studies, which are performed in crude muscle extracts, do not take into account fibre type-specific effects, and therefore age-related changes in a specific enzyme could be partially due to different rates of fibre degeneration and not just because of altered expression, modification and/or degradation of this enzyme.

Oxidative stress

Accumulating evidence suggests that oxidative stress underlies the ageing process in skeletal muscle. Proteomic analysis of muscle ageing identified enzymes implicated in scavenging of reactive oxygen, as well as enzymes challenged with the detoxification of cytotoxic compounds produced by oxidative stress. Oxidative stress increases the production of cytotoxic aldehydes, which can react with cellular proteins, nucleic acids, and cell membranes. Evidence for increased detoxification of cytotoxic products in old muscle has been provided by the differential regulation of mitochondrial aldehyde dehydrogenase and glutathione transferase (Piec et al. 2005; Lombardi et al. 2009). Another feature of muscle ageing is the up-regulation of molecular chaperones, which detect, refold (Hsp20, Hsp27, Hsp70, Hsp90) and eventually eliminate (ER60) abnormal proteins (Doran et al. 2007; Lombardi et al. 2009; Staunton et al. 2012). For example, alpha-B crystallin, which is increased in old muscle serves as a chaperone for desmin preventing its aggregation under various forms of stress (Goldfarb and Dalakas 2009). The up-regulation of a subpopulation of Hsps may then play a major role in preventing deleterious protein aggregation, and may also be of general importance for the maintenance of cellular integrity during the age-induced remodelling of muscle myofibres. In addition to the differential expression of proteins of the contractile networks, energy metabolism, and stress response, proteome analyses have identified differentially expressed proteins that are potentially important for muscle ageing. These include several carriers (carbonic anhydrase 3, myoglobin, or vitamin D binding protein) involved in metabolite transport (Doran et al. 2008; Staunton et al. 2012), and a few of the components (14-3-3 protein, protein phosphatase 2A) of the signal transduction pathways (Piec et al. 2005; Capitanio et al. 2009).

Secretome of muscle cells

During the last 5 years, several in vitro studies have characterized the secretome profiles of muscle cells, such as C_2C_{12} myotubes (Chan et al. 2011; Henningsen et al. 2010), human myotubes (Le Bihan et al. 2012) and rat muscle explants (Roca-Rivada et al. 2012) using either shotgun or 2D gel strategies, and have demonstrated the capacity of muscle cells to secrete growth factors (e.g. follistatin like protein 1, IGF2, TGF), cytokines, and inhibitors of collagenase (e.g. TIMP2). Secreted proteins—also named myokines (Engler 2007)—may act in an autocrine/paracrine manner on muscle cells or other cell types and contribute to muscle growth and regeneration, body-wide metabolism, as well as other functions [see (Yoon et al. 2012) for review]. In addition to proteins exiting the cell by classical secretory pathways, human and murine muscle cells also release protein-associated vesicles (Le Bihan et al. 2012; Duguez et al. 2013). Two distinct categories of vesicles have been identified in the culture medium of human myotubes:

(1) exosomal-like nanovesicles with a cup-shape, and (2) polymorph microvesicles with electron dense cores. The exosome-like population is enriched with plasma membrane, sub-plasma membrane, endosome and lysosome proteins, whereas microvesicles are preferentially enriched with endoplasmic reticulum, mitochondrial, golgi, cytoskeletal, and cytosol proteins (Le Bihan et al. 2012). In addition, both types of vesicles can fuse and deliver functional proteins into target cells, as shown by the delivery of alkaline phosphatase through vesicles to human dermofibroblast cells that do not have an endogenous activity for alkaline phosphatase (Le Bihan et al. 2012), and might thus play a key role in the cell–cell communication.

The secretion capacity of myoblasts or differentiating myotubes from aged patients has never been investigated. Our preliminary analysis of the transcriptome of differentiated myoblasts from old and young adult donor revealed that a third of the genes that are misregulated are genes related to secreted or membrane proteins (Fig. 2a), suggesting that the secretome of elderly muscle might be altered and could affect the myofiber environment. This altered secretome might have an impact on the metabolism of neighbouring myofibers, on innervation, and on the behavior of nearby cells such as myoblasts, fibroblasts and adipocytes (Fig. 2b). A better characterization of the changes of the secretome with ageing might help us to understand the orchestration of the complex processes involved in sarcopenia.

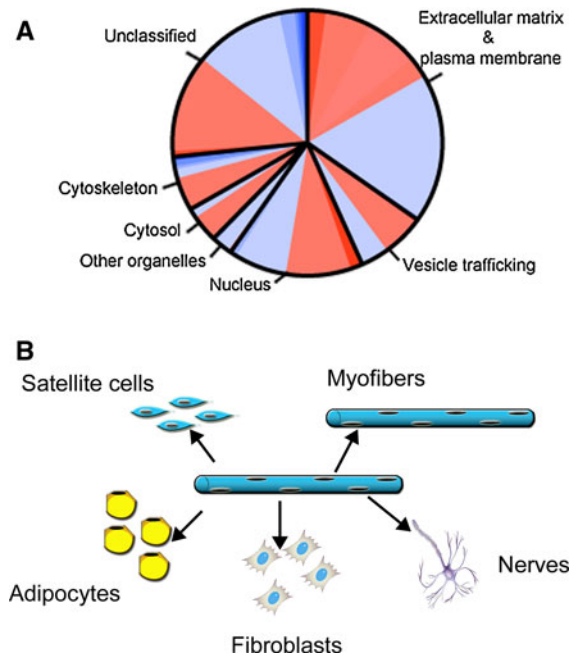

Fig. 2 Changes in levels of secreted proteins with ageing: impact on myotube environment. **a** Transcriptome analysis of differentiated myoblasts extracted from young and elderly subject. 271 genes were found differentially expressed. 94 genes are encoding for extracellular or membrane proteins, and 23 genes for proteins involved in vesicle trafficking. **b** The myotube secretome might interfere with the balance of protein synthesis/degradation of nearby myofibers, or with the innervation, and thus stimulate myofiber atrophy. It might also have an impact on the behaviour of nearby cells such as fibroblasts, myoblasts and adipocytes and thus decrease the potency to repair damaged fibers and stimulate the deposition of fibrotic and fatty tissue

Oxidative proteome modifications during skeletal muscle ageing

Skeletal muscle and reactive oxygen species

Although oxidative damage to cellular macromolecules may contribute to tissue dysfunction, there has been increasing recognition that ROS will also mediate physiologic processes in tissues. ROS interact with multiple cell signalling and regulatory pathways to modulate changes in gene expression and are recognized to regulate a number of physiological processes in skeletal muscle by acting as second messengers in cellular signal transduction pathways (Droge 2002; Jackson et al. 2002). It should be emphasized that skeletal muscle fibers possess strong antioxidant systems that protect cells from potential ROS-induced deleterious effects. For instance, the antioxidants CuZn-superoxide dismutase (SOD), catalase, and glutathione peroxidase are present in the sarcoplasm, whereas Mn-SOD and glutathione peroxidase are localized within the mitochondrial matrix (Barreiro and Hussain 2010). Other thiol-based antioxidant proteins, such as thioredoxins and peroxiredoxins, are also abundantly expressed inside skeletal muscle fibers (Ferreira and Reid 2008). However, increases in the production of ROS to levels that are significantly greater than those that can be neutralized by intracellular antioxidant defences will lead to the development of a state of oxidative stress in the skeletal muscle, which will have profound effects on action-potential conduction, excitation–contraction coupling, satellite cell differentiation, contractile proteins, and mitochondrial respiration (Reid 2001).

Elevated levels of ROS generated inside skeletal muscle fibers in response to proinflammatory conditions have also been shown to cause a severe decrease in muscle contractile performance (Shindoh et al. 1992). ROS mediated damage has also been proposed to be involved in disuse muscle atrophy. Kondo et al. (1991), have shown that immobilization of skeletal muscles is associated with increased ROS production resulting in oxidative injury leading to inactive muscle fibers, and that disuse muscle atrophy could be retarded via antioxidants (Kondo et al. 1994). These observations have subsequently been confirmed and it is now clear that oxidative stress can contribute to the rate of muscle atrophy during sarcopenia (Powers and Jackson 2008). Although it is believed that oxidative stress mediates skeletal muscle dysfunction through macromolecular damage, the molecular mechanisms remain elusive.

The main sources of ROS generation inside skeletal muscle fibers are the mitochondria, xanthine oxidase and NADPH oxidases. ROS generation by the mitochondria is elevated inside skeletal muscle fibers and therefore might be the origin of macromolecular damage. Another important source of ROS generation inside skeletal muscle fibers is xanthine oxidase. Under physiological conditions, hypoxanthine and xanthine are oxidized to uric acid by xanthine dehydrogenase. However, under ischemic or hypoxic conditions, xanthine dehydrogenase is converted to xanthine oxidase, which preferentially reduces molecular O_2 to H_2O_2 (Engerson et al. 1987). During stress, both hypoxia and increased levels of xanthine and hypoxanthine cause significant elevation of xanthine oxidase activity, which may also contribute to elevated levels of ROS in skeletal muscle. Moreover, recent studies suggest that xanthine oxidase-derived ROS contribute significantly to oxidative protein modifications, including carbonyl formation inside skeletal muscle fibers (Whidden et al. 2009).

Protein oxidation and related modifications

Proteins represent the main targets for ROS mediated damage that occurs either directly or indirectly through their reaction with lipids and carbohydrates and the subsequent generation of oxidized products that can react with proteins. Protein oxidation by ROS can be classified into those that oxidize and cleave the peptide bond and those that oxidize the side chains. Almost all amino acid side chains can react with the hydroxyl radical (*OH), but certain amino acids are more sensitive to oxidation. Indeed, all sorts of ROS readily oxidize the sulfur-containing amino acids methionine and cysteine. Oxidation products of cysteine include disulfide bridges and sulfenic acid that can be converted to disulfide bridges or further oxidized to sulfinic and then sulfonic acids. Both disulfide bridges and sulfenic acid can be enzymatically reduced while sulfinic acid reduction has so far been limited to oxidized cysteines within the active site of peroxiredoxins (Biteau et al. 2003; Rabilloud et al. 2002). Cysteine can also react with nitric oxide to produce *S*-nitrosothiol (SNO). Methionine oxidation leads to the formation of methionine sulfoxide and further oxidation of methionine sulfoxide leads to the irreversible formation of methionine sulfone. In addition, some oxidative modifications are quite specific in terms of oxidized residues and products generated such as the oxidation of phenylalanine to tyrosine, which can be further converted to di-tyrosine (Giulivi et al. 2003). Moreover, tyrosine residues represent preferred targets for nitration by nitrogen dioxide and peroxynitrite and can be converted to nitrotyrosine (Fugere et al. 2006).

On the other hand, oxidation of several amino acid residues such as lysine, arginine, proline and threonine results in the formation of carbonyl groups (Berlett and Stadtman 1997). Carbonyl derivatives can also originate from the fragmentation products of the peptide bond oxidative cleavage (Stadtman and Levine 2003). Aminoadipic and glutamic semi-aldehydes resulting from the oxidation of lysine and arginine, respectively, are quantitatively important products of the carbonylation reaction. In addition to direct oxidation, protein carbonyl derivatives can originate from the conjugation on cysteine, lysine and histidine residues with aldehydes as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE). Indeed, oxygen free radicals can attack cellular membranes and induce lipid peroxidation resulting in the production of these reactive aldehydes which are precursors of advanced lipid peroxidation end products that have been found to accumulate on proteins during ageing and certain age-related diseases (Moreau et al. 2003). Moreover, sugar aldehydes or ketones can also react spontaneously with the amino groups of lysine and arginine through the formation of a Schiff base which is slowly rearranged to form an Amadori product (e.g.

fructosamine when the reacting sugar is glucose). These products are referred as to early stage glycation adducts that are further modified to form stable end-stage products also called advanced glycation end products (AGE) through either rearrangement, oxidation, dehydration, fragmentation and/or cyclization. Deleterious effect on protein function is observed when the modification affects critical amino acids within the protein and many proteins, including intracellular proteins (Horiuchi and Araki 1994).

Although an increased load of oxidatively modified proteins has been clearly associated with normal and pathological ageing, in most cases the target proteins have not been identified and, only recently significant advances have been made towards the identification of proteins targeted by these modifications. Indeed, identification of these proteins (the "Oxi-Proteome"), the modified forms of which are accumulating during ageing or upon the development of an age-related disease, would be expected to give some insights into the mechanisms by which these damaged proteins would build-up and potentially affect protein function. Moreover, although the causative role of protein oxidative modifications has not yet been determined, the accumulation of oxidatively damaged proteins during ageing and their particular increase in organs and tissues affected by age-related diseases imply that the restricted set of proteins targeted by damage may be a potential substratum for many of the observed cellular dysfunction. Protein carbonylation has been considered as an indicator of severe oxidative damage as well as age- and disease-derived protein dysfunction since it often leads to a loss of protein function, as well as an increased thermosensitivity or hydrophobicity of the targeted protein (Berlett and Stadtman 1997; Baraibar et al. 2012b).

Previous studies have addressed in mammalian models the importance of protein damage in the development of sarcopenia (Thompson et al. 2006; Snow et al. 2007; Choksi and Papaconstantinou 2008; Andersson et al. 2011).

In humans, preliminary studies on skeletal muscle have shown increasing amounts of protein carbonyl content during ageing (Pansarasa et al. 2000). Muscles with different functions in humans have been compared (Marzani et al. 2005), and some authors have reported an increase in carbonylated proteins during ageing in both the *vastus lateralis* (Gianni et al. 2004) and the *external intercostal* (Barreiro et al. 2006). However, the specific targets of oxidation have not been identified, thus future studies should approach this important issue. We have recently generated a database of proteins which have been identified as increasingly carbonylated or modified by AGE or HNE during ageing and age-related diseases in different tissues and organ systems, such as brain, cerebellum, spinal cord, skeletal muscle, liver, eye, and cerebrospinal and bronchoalveolar fluids (Baraibar et al. 2012a). Several modified proteins have been consistently identified in the different organs, indicating that the spectrum of proteins targeted by these modifications may be conserved. Among them, cytoplasmic proteins were predominant, followed by proteins from mitochondria, nucleus, endoplasmic reticulum and plasma membrane. Functional annotation indicated that proteins were mainly distributed within biological processes such as inflammatory response, cellular metabolism, free radical scavenging, protein synthesis and folding. Proteins involved in energy metabolism were also evidenced in the modified proteins referenced. The most significant canonical pathways across the entire dataset included: glycolysis/gluconeogenesis, citrate cycle, pyruvate metabolism, amino acids degradation, mitochondrial dysfunction, cell death, butanoate metabolism, nrf-2 oxidative stress response and cellular function and maintenance (Baraibar and Friguet 2013). Taken together, these observations suggest that important cellular functions, like energy production, carbohydrate metabolism, protein synthesis, folding and degradation may be affected by modification of key proteins involved in these pathways. Common processes are suggested to be causally involved or at least contribute to ageing and age-related diseases, including increased oxidative stress, accumulation of protein damage and general metabolic dysfunction. However, these processes have been mostly seen as independent events. Different set of data indicate that several enzymes that catalyze intermediate metabolism, such as glycolysis and gluconeogenesis, the citrate cycle and fatty acid metabolism are particularly targeted by deleterious modifications (Baraibar and Friguet 2013). These results indicate a potential effect of protein modification on the impairment of cellular energy metabolism. Future studies should address this important issue in skeletal muscle, for instance by combining metabolomics, targeted modification proteomic analysis and functional proteomics.

Detection, quantification and identification of oxidatively damaged proteins

Since protein carbonyls are the most commonly used marker of protein oxidation, different methods have been developed for the detection and quantification of carbonylated proteins. Most of them are based on immunochemical and/or spectrophotometric assays of protein carbonyls previously derivatized with 2-4-dinitrophenylhydrazine (DNPH) to form 2-4-dinitrophenylhydrazone (DNP) proteins adducts (Levine et al. 2000). In turn, this approach is based on the ability of DNPH to bind covalently to the carbonyl groups (through a Schiff base) to allow formation of stable hydrazones. Total carbonyl groups on a specific protein or in a mixture of proteins can be detected and quantified spectrophotometrically due to the DNP characteristic absorption spectrum with a maximum at 365–375 nm. Given the commercial availability of antibodies against DNP, the detection of total carbonyl groups can be also performed by western blot, dot blot or ELISA (after derivatization of carbonyl groups to DNP). Although an increased load of carbonylated proteins has been clearly associated with ageing, in most cases the target proteins have not been identified and are one of the main limitations of the above mentioned techniques. Global approaches, such as proteomics, are at present the best because they provide comparative information on the constant remodelling of the cell proteome through its biochemical interactions with the genome and the environment. Thereby, proteomics is useful for the identification of carbonylated proteins in a complex mixture (Rogowska-Wrzesinska et al. 2013). Moreover, identification of such modified proteins may also help to identify how these damaged proteins are accumulated during the ageing process or in disease conditions. For this purpose it is necessary to separate proteins by 2D electrophoresis or to enrich carbonylated peptides (i.e. by affinity chromatography) before MS analyses. Despite the limitations of 2D gel electrophoresis, it is still considered as one of the best technique for separation of a complex mixture of soluble proteins before carbonyl detection. The use of 2D gels has proved very useful for the detection of specific carbonylated protein spots, especially when comparing two physiological conditions (Ahmed et al. 2010; Baraibar et al. 2011). Although absolute carbonyl quantification is a limitation either by gel based or gel free proteomic approaches, 2D separation of proteins has the advantage of showing the images obtained after detection of the functional group attached to the carbonyl group as well as those obtained after staining the gels with dyes for total proteins, such as SYPRO Ruby and Coomassie Brilliant blue. This allows the normalization of the carbonyl signal to the amount of protein determining the relative modification index for each spot. This is an important point because, as in any proteomic analysis, the study of carbonylated proteins is biased in favour of the most abundant proteins.

Several authors have also explored gel-free methods based on affinity enrichment of carbonylated proteins and subsequent detection by MS (Fedorova et al. 2010; Madian and Regnier 2010). However, the disadvantage of these approaches is that they do not allow a quantitative analysis of the degree of carbonylation of the identified proteins, which limits their usefulness for studying changes in the pattern of protein oxidation under different biological conditions. The most common system for the enrichment of carbonylated proteins uses the avidin–biotinhydrazide system. The enriched proteins are digested and the resulting peptides are identified by LC–MS/MS. Due to the limitations mentioned above, alternatives for carbonylated protein detection and quantification are emerging.

The specificity of hydrazide derivatives to carbonyl groups and the presence of a wide range of functional groups coupled to the hydrazide, allow the development of strategies for the detection and quantification of carbonylated proteins (Yan and Forster 2011). In addition, labelling of proteins with fluorescent dyes has been extensively applied in proteomic investigations. A technique merging these two characteristics would be of great value. Fluorescence difference in-gel electrophoresis, usually abbreviated as DIGE (Ünlü and Minden 2002), would seem to be the appropriate technique due to its potential to directly compare proteins from two different groups of samples. This advanced gel electrophoretic method represents one of the most powerful analytical tools for conducting comparative protein biochemical investigation. The DIGE technique is an ideal method for comparing entire soluble proteomes in one swift analytical approach DIGE greatly reduces gel-to-gel variations and thereby greatly improves the evaluation of trends in modifications observed in protein

expression patterns (Ünlü and Minden 2002). Analytical DIGE systems can be employed with two-dye or three-dye systems, depending on the specific application. DIGE analysis, using an internal pooled standard, is a highly accurate quantitative method that enables multiple protein samples to be separated on the same 2D gel.

Recently, a novel application of the DIGE approach has been developed, but for the detection and quantification of carbonylated proteins, referred as: Oxi-DIGE (Baraibar et al. 2012c). In Oxi-DIGE, protein carbonyls are labelled with fluorescent hydrazide probes that bind specifically to carbonyl groups in proteins. Following oxidative stress, protein extracts from human myoblasts (LHCNM2) were analyzed. Protein carbonyls were labelled with the CyDyeTM hydrazides (GE Healthcare). Labelled protein extracts from stressed and control cells were mixed together and separated by 2D electrophoresis in the same gel. Importantly, in Oxi-DIGE two samples from different groups (in this example control and stressed) are co-migrated on a single 2D gel for direct quantification, without using antibodies. Carbonylated proteins that are present in both samples will form coincident spots, evidenced by the merge of the two colours used for labelling the different samples. Carbonylated proteins that are not present in both samples will migrate independently and will be evidenced by the specific colour used for labelling one or the other sample. An internal standard was used by mixing equal amounts of the eight above-mentioned samples and labelled with CyDyeTM Cy2 *N*-hydroxysuccinimide. In addition the inclusion of a pooled-sample mixture as an internal standard (for a series of coordinated Oxi-DIGE gels) allows for a more reliable and statistically powerful experimental design that provide increased statistical confidence. In addition, with this technique it is now possible to superimpose the oxi-proteome with the total proteome of the same sample on a single gel (Fig. 3).

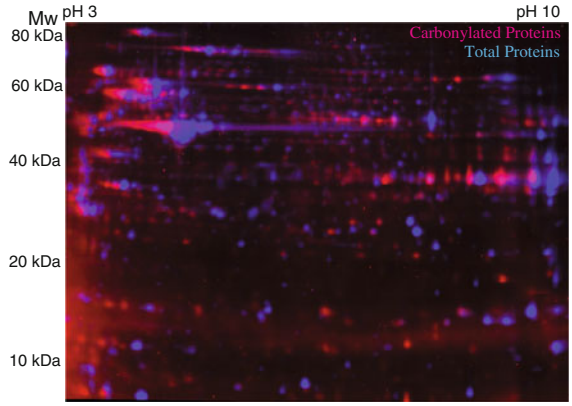

Fig. 3 2D map of the oxi-proteome and total proteome of LHCNM2 human myoblasts upon oxidative stress. After hydrogen peroxide induced oxidative stress (1 h, 500 μM) to LHCNM2 myoblasts, cellular pellets were homogenized in a lysis buffer and clarified by centrifugation. Protein carbonyls and total proteins were labeled with CyDyeTM hydrazide (GE Healthcare) or CyDyesTM N-hydroxysuccinimide (GE Healthcare) as previously reported (Baraibar et al. 2012c). After labeling, 100 μg of protein were electro-focused on 24 cm pH 3–10 non-linear isoelectrofocusing strips and then separated by 8–20 % gradient SDS-PAGE. Analytical gels were imaged using an Ettan DIGE imager (GE Healthcare) scanner. *Blue spots* total proteins, *Magenta spots* carbonylated proteins. (Color figure online)

Oxidized protein pattern generated upon oxidative stress in human myoblasts

In recent years particular interest has arisen concerning the effects of oxidative stress on human stem cells, the consequences of this on their differentiation capacity and its potential impact on tissue regeneration. We have recently characterized the proteome changes in adult human muscle stem cells in response to oxidative stress (Baraibar et al. 2011). Using a dual proteomic approach, we have begun to unravel the mechanism involved in human myoblast dysfunction upon oxidative stress. Selective proteins either modulated at the expression level or those targeted by oxidation (carbonylated) were identified after a sub-toxic exposure to hydrogen peroxide, which induced intermediate cellular damage without suppressing the myoblasts anti-oxidant response mechanisms. Although cell viability was not compromised, at least in the 24 h after the insult, increased carbonylation of proteins was observed (Baraibar et al. 2011). Since intracellular accumulation of carbonylated proteins results in deleterious effects on cell function and survival, the identification of those proteins specifically targeted by oxidation is of valuable interest. For this purpose, a 2D gel electrophoresis-based proteomic approach coupled with immunodetection of carbonylated proteins, after their derivatization with DNPH, and identification of the spots of interest by MS was used. Twenty-one protein spots were identified as being increasingly carbonylated upon oxidative

stress, indicating that only a restricted set of proteins is prone to accumulate modifications upon oxidative stress. Most of the carbonylated proteins identified were localized in the cytosol, the nucleus, the endoplasmic reticulum as well as the plasma membrane. Major functional categories include energy metabolism, cellular assembly, protein synthesis, cell morphology and protein degradation. Since protein carbonylation is an irreversible modification, the cell must degrade the damaged proteins and trigger de novo synthesis in order to maintain a healthy protein pool and reduce the accumulation of potentially toxic proteins. However, proteins involved either in protein degradation such as the proteasome regulatory subunit 10B, and in protein synthesis such as elongation factor 2 were found to be carbonylated. Previous studies have shown that protein synthesis is inhibited by oxidative stress (Shenton et al. 2006). Furthermore, carbonylation of the proteasome subunits may explain, at least in part, the decreased proteasome activity observed, suggesting that oxidative stress not only induces protein modification, but also compromises their degradation by affecting proteasome function.

Conclusions

In summary, although it is recognized that cellular ageing causes changes in the proteome, the nature and targets of these changes and their consequences on skeletal muscle and how they may contribute to sarcopenia have not yet been completely elucidated. In this context, 2D-gel electrophoresis based proteomic approaches represent powerful tools to address these questions by monitoring at the proteome level both the quantitative changes of protein content and the extent of protein oxidative modifications and by identifying the targeted proteins. This information is essential in order to obtain further insights into the metabolic pathways that may be altered during muscle ageing and in situations of oxidative stress. Previous studies have suggested that changes in protein expression and/or oxidative modifications contribute to the impairment of energy metabolism, cytoskeleton, stress response and protein quality control. Interestingly, all these metabolic pathways have already been implicated in ageing of different organisms and organ system. Future studies should address this important issue for instance by combining metabolomics and targeted proteomic analysis during myoblast senescence and skeletal muscle ageing.

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Publication 7

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Apoptosis in capillary endothelial cells in ageing skeletal muscle

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Summary

The age-related loss of skeletal muscle mass and function (sarcopenia) is a consistent hallmark of ageing. Apoptosis plays an important role in muscle atrophy, and the intent of this study was to specify whether apoptosis is restricted to myofibre nuclei (myonuclei) or occurs in satellite cells or stromal cells of extracellular matrix (ECM). Sarcopenia in mouse gastrocnemius muscle was characterized by myofibre atrophy, oxidative type grouping, delocalization of myonuclei and ECM fibrosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) indicated a sharp rise in apoptosis during ageing. TUNEL coupled with immunostaining for dystrophin, paired box protein-7 (Pax7) or laminin-2α, respectively, was used to identify apoptosis in myonuclei, satellite cells and stromal cells. In adult muscle, apoptosis was not detected in myofibres, but was restricted to stromal cells. Moreover, the age-related rise in apoptotic nuclei was essentially due to stromal cells. Myofibre-associated apoptosis nevertheless occurred in old muscle, but represented < 20% of the total muscle apoptosis. Specifically, apoptosis in old muscle affected a small proportion (0.8%) of the myonuclei, but a large part (46%) of the Pax7$^+$ satellite cells. TUNEL coupled with CD31 immunostaining further attributed stromal apoptosis to capillary endothelial cells. Age-dependent rise in apoptotic capillary endothelial cells was concomitant with altered levels of key angiogenic regulators, perlecan and a perlecan domain V (endorepellin) proteolytic product. Collectively, our results indicate that sarcopenia is associated with apoptosis of satellite cells and impairment of capillary functions, which is likely to contribute to the decline in muscle mass and functionality during ageing.

Key words: angiogenesis; apoptosis; sarcopenia; satellite stem cell.

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Introduction

Sarcopenia is the progressive generalized loss of skeletal muscle mass and function, which occurs as a consequence of ageing. Sarcopenia results in impaired locomotion and is associated with an increased susceptibility to illness, as muscle is a major site of fatty acid oxidation and carbohydrate metabolism and a body reservoir of readily available amino acids. Multiple phenomena are involved in the development of sarcopenia. In humans, intrinsic factors include altered hormonal levels (menopause, andropause, adrenopause, somatopause), increased levels of inflammatory cytokines (Lamberts *et al.*, 1997), neuronal remodelling (Lexell, 1997), fibrosis of the extracellular matrix (ECM) (Kragstrup *et al.*, 2011), and impaired microvascular (Herrera *et al.*, 2010) and satellite cell functions (Renault *et al.*, 2002). Extrinsic factors such as a poor nutritional status or physical activity also play major roles in the aetiology of sarcopenia (Valdez *et al.*, 2010).

From a histological perspective, muscle ageing is characterized by a decrease in myofibre size and number, with a preferential loss of type II myofibres. At the myofibrillar level, important modifications in contractile and cytoskeletal components, and in essential regulatory proteins, likely account for dysfunctions in old muscle contraction, as shown in rodents (Piec *et al.*, 2005). Other features support perturbations in protein turnover (Combaret *et al.*, 2009), reduced energy metabolism and altered detoxification of reactive oxygen species (Baraibar *et al.*, 2013). Obviously, the mechanisms relating to sarcopenia are complex and probably result from the alteration of a variety of interrelated cellular functions (Ibebunjo *et al.*, 2013).

Although more information is available about the mechanisms that affect contractile myofibres during ageing, few studies have investigated the implication of ECM embedding myofibres. ECM is a critical component in the transfer of force from the muscle myofibre out to the tendon and subsequent bone (Voermans *et al.*, 2008). ECM plays an important role in maintaining the structure of the muscle and also in providing an environment in which the contractile myofibres can function. ECM also contains different types of stromal cells such as fibroblasts, immune cells, adipocytes and capillary cells, which reciprocally are involved in the turnover of ECM and in the regulation of myofibre metabolism (Kragstrup *et al.*, 2011).

Recent evidence indicates that apoptosis is involved in mediating the progression of sarcopenia (Marzetti *et al.*, 2010). There is also increasing knowledge about the pathways and effectors of the apoptotic process in skeletal muscle. However, it is not fully understood whether age-dependent apoptosis is mainly restricted to myofibre nuclei (myonuclei) or whether apoptosis also occurs in other muscle cells. Herein, we report that apoptosis in old skeletal muscle is not confined to myofibres, but is preponderant in capillary endothelial cells and of significant importance in satellite cells. We further provide evidence that ECM remodelling is characterized by a limited proteolysis of the basement membrane perlecan and with the production of endorepellin fragments.

Results

Age-dependent modifications in myofibre morphology

The preferential loss of *gastrocnemius* muscle (GM) muscle mass is shown in Fig. 1. Body weight increased during maturation, between 2

Aging Cell

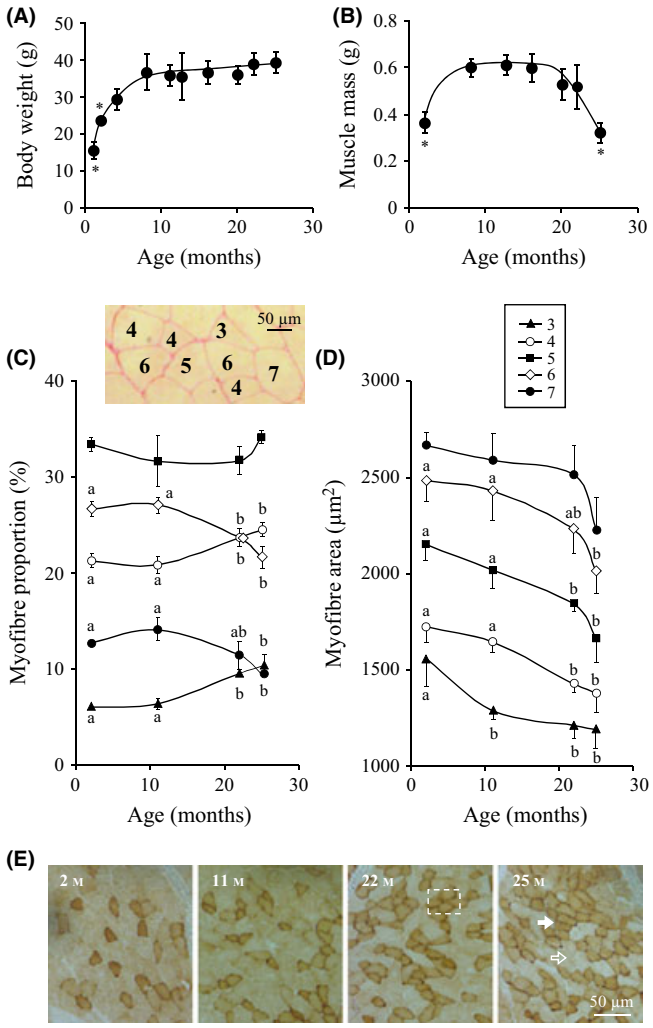

Fig. 1 Sarcopenia in C57BL6 mice and myofibre morphology. (A) Body weight and (B) *gastrocnemius* muscle (GM) mass for 2–25 months old C57BL6 mice. Muscle cross sections were stained with Sirius red, and 800 individual myofibres were analysed per mouse and classified according to the number of neighbour myofibres. Age-dependent changes in the (C) distribution and (D) cross-sectional area of myofibres with 3, 4, 5, 6 or 7 neighbours in 2–25 months GM. All values in the graphs are means ± SEM (vertical bars) for $n = 4$ mice per age, and different letters indicate significant difference ($P < 0.05$) between ages. (E) Images of GM cross sections stained for cytochrome oxidase (Cox) in 2–25 months mice. Cox-positive (solid arrow) and Cox-negative (hollow arrow) myofibres are indicated. Region of interest (dotted box) in 22 months muscle cross section highlights oxidative myofibre grouping.

and 11 months, and then remained stable until 25 months (Fig. 1A). GM mass similarly raised during maturation was maintained in adult mice, but strongly decreased during ageing (from 20 to 25 months) (Fig. 1B). This marked atrophy of GM muscle reflected sarcopenia in old mice.

Age-dependent muscle atrophy was associated with modifications in myofibre morphology, so that old myofibres appeared less polygonal, that is, surrounded by less neighbouring myofibres. To quantify this observation, we classified myofibres according to their number of neighbours and measured cross-sectional areas for about 800 individual myofibres per mouse. In the young adult (2 months), mature adult (11 months), early old (22 months) and advanced old (25 months) GM, myofibres with five neighbours were always the most abundant. However, during ageing, the proportion of myofibres with six and seven neighbours decreased, while symmetrically the myofibres with three and four neighbours increased, suggesting that myofibres became more acute in shape (Fig. 1C). As expected, larger myofibres had more neighbours, and as shown in Fig. 1(D), the most abundant classes of myofibres (four to six neighbours) exhibited an age-related decrease in cross-sectional area.

Cytochrome c oxidase (Cox), a marker for oxidative energy metabolism, characterizes slow contracting myofibres. In mice GM, Cox preferentially labelled the small myofibres with three to five neighbours. As shown in Fig. 1(E), Cox myofibres presented a chessboard-like distribution in muscle cross sections of 2 and 11 months adult muscles, while myofibre-type grouping was clearly apparent in 22 and 25 months old muscles. Therefore, GM ageing in mice was associated with atrophy and grouping of acute-shaped myofibres.

Age-dependent modifications in the various populations of muscle nuclei

Skeletal muscles contain different cellular populations: multinucleated myofibres, satellite cells and stromal cells of ECM. Stromal cells are located outside the basal lamina, while most satellite cells are located between the myofibre sarcolemma and basal lamina (Scharner & Zammit, 2011). Further studies were then performed to specify which cellular population is mainly affected by ageing.

Hoechst staining of nuclei and colabelling of the sarcolemma with anti-dystrophin were used to distinguish nuclei in myofibres (myonuclei; Fig. 2A). These analyses indicated that myofibres maintained a similar content of myonuclei with age ($P > 0.42$). However, because of smaller cross-sectional area, the myonuclear domain (the myofibre area controlled per myonucleus) significantly decreased in 25 months muscles (Fig. 2B; $P < 0.03$). While myonuclei are typically located beneath the sarcolemma in young and adult myofibres, a characteristic feature of the old muscle was also a sharp rise in centrally located myonuclei (Fig. 2C; $P < 0.001$). Hoechst staining of total muscle nuclei and costaining of the basal lamina further indicated that, outside the basal lamina, stromal cell nuclei (Fig. 2A, arrow head) represented a significant proportion of total muscle nuclei. However, this proportion of stromal nuclei remained unchanged during ageing (48.6 ± 3.2 vs. 44.7 ± 2.8% in 11 and 25 months muscles, respectively, $P = 0.25$).

The nuclear transcription factor paired box protein-7 (Pax7) is a reliable marker of both quiescent and activated satellite cells (Péault *et al.*, 2007), and anti-Pax7 was then used to investigate these muscle stem cells. Triple labelling with Hoechst, anti-Pax7 and anti-laminin 2α confirmed that $Pax7^+$ satellite cell nuclei were located beneath the basal lamina (Fig. 2D). Our study of $Pax7^+$ cells in GM cross sections from young adult to old mice indicated that the proportion of satellite cells strongly decreased (−51%; $P < 0.05$) during maturation (2–11 months), but remained at a low level thereafter in adult and old muscles (Fig. 2E).

Apoptosis essentially occurs in extracellular matrix stromal cells

Because the modifications that occur in muscle during ageing might be related to apoptosis, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) analyses. As shown in Fig. 3(A), $TUNEL^+$ nuclei strongly increased in the old muscle ($P < 0.001$). To further distinguish whether apoptosis occurred in myofibres or in

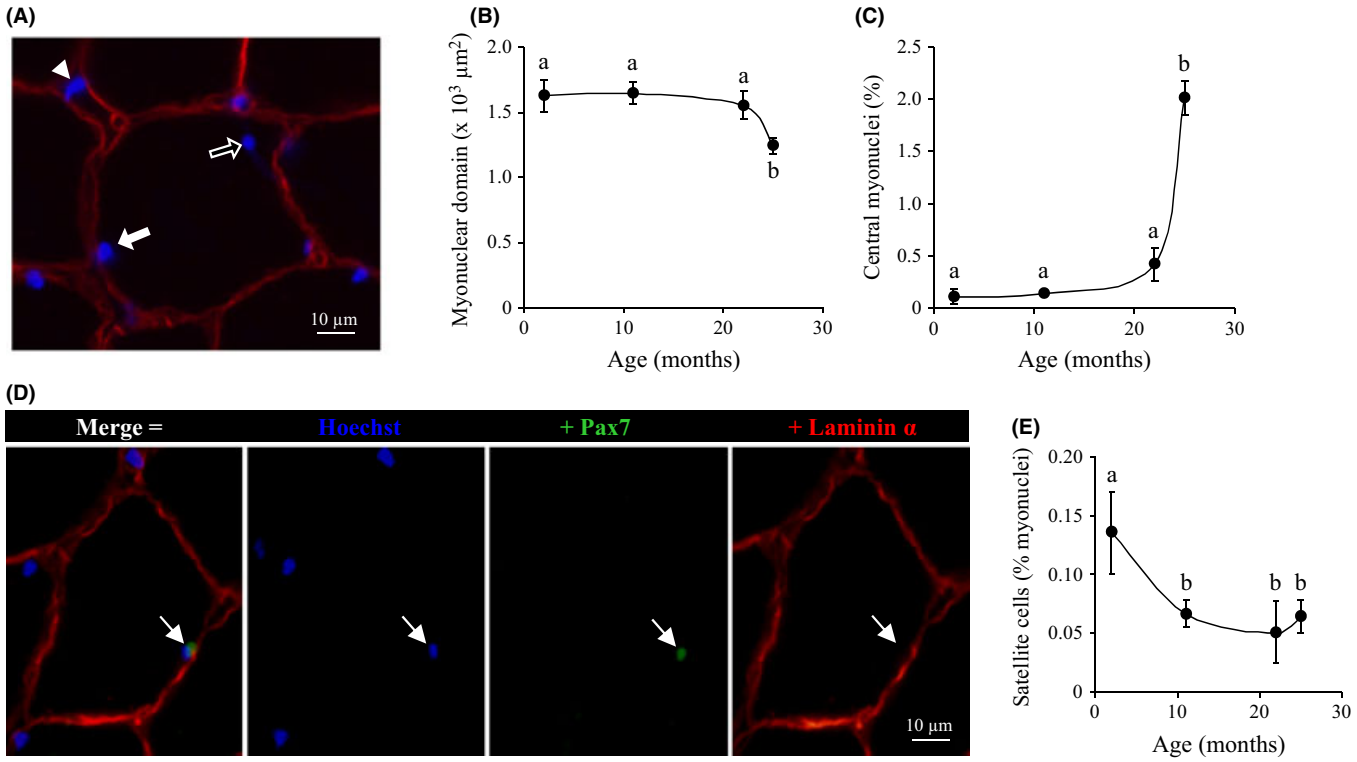

Fig. 2 Myofibre nuclei during ageing of mouse muscle. (A) Representative image of a 25 months muscle cross section labelled for nuclei (Hoechst 33258, blue) and plasma membrane dystrophin (red). A peripheral myonucleus (solid arrow), internal myonucleus (hallow arrow) and stromal cell nucleus (arrow head) are indicated. Age-related variations in (B) myonuclear domain (myofibre area controlled per myonucleus), and (C) proportion of internal myonuclei. (D) Immunolocalization of a satellite cell in a 2 months muscle cross section colabelled with Hoechst 3258 (blue), anti-paired box protein-7 (Pax7) (satellite cells, green) and anti-laminin 2α (basal lamina, red). (E) Proportion of satellite cell nuclei among total myofibre nuclei. Values in the graphs are means ± SEM (n = 4), and different letters indicate significant difference ($P < 0.05$) between ages.

ECM stromal cells, immunostaining of laminin 2α was performed together with TUNEL and Hoechst (Fig. 3B). Interestingly, apoptotic nuclei in adult mice essentially belonged to stromal cells in the connective tissue. Moreover, the age-dependent rise in apoptotic nuclei was mostly attributed to stromal cells (Fig. 3A, white bars). TUNEL studies coupled with Hoechst and laminin 2α immunostaining nonetheless indicated that apoptosis also occurred in myofibre-associated nuclei (myonuclei and/or satellite nuclei), but only in old muscles (< 20% of total muscle apoptosis; Fig. 3A, black bars).

Apoptosis in myofibres and in satellite cells

Further studies were then performed with 25 months muscles to specify whether myofibre-associated apoptosis was due to myonuclei and/or to satellite cells. Muscle cross sections were immunolabelled for dystrophin to specifically localize the sarcolemma and to distinguish myonuclei (inside the sarcolemma), from satellite and stromal nuclei (outside the sarcolemma) (Fig. 3C). Triple labelling with TUNEL, Hoechst and anti-dystrophin of GM cross sections was carried out for four old mice. More than 1200 myofibres were assessed per mouse, but apoptosis was found to affect only a limited proportion (0.78 ± 0.15%, n = 4) of the total myofibre myonuclei.

To identify apoptotic satellite cells, cross sections of 25 months muscle were triple labelled with TUNEL, Hoechst and anti-Pax7 (Fig. 3D). Of the 17 satellite cell nuclei identified in the old GM of four mice, eight were found to be apoptotic. Therefore, our study revealed the existence of TUNEL$^+$ satellite cells and strikingly that apoptosis occurred for a significant proportion (45.8 ± 6.7%, n = 4) of the Pax7$^+$ satellite cells in GM from 25 months old mice.

Apoptosis in capillary endothelial cells

Because TUNEL-positive nuclei were predominantly found in stromal cells in adult and old GM, additional investigations were carried out to identify which cellular population is apoptotic in ECM. ECM contains different categories of stromal cells, including fibroblasts, adipocytes, capillary cells and immune cells. Macrophages or other white blood cells could rarely be observed in our muscle cross sections. CD31 is highly expressed on endothelial cells and is a major constituent of the intercellular junction in confluent vascular beds (Privratsky *et al.*, 2010). Anti-CD31 is therefore commonly used to mark capillary endothelial cells (Christov *et al.*, 2007). Triple labelling of muscle cross sections with TUNEL, Hoechst and CD31 was then used to label apoptotic nuclei, total nuclei and capillary endothelial cells, respectively (Fig. 4A). These studies indicated that in adult GM, a significant proportion of the apoptotic stromal cells were CD31$^+$ capillary endothelial cells (Fig. 4B). Moreover, our data revealed that the age-dependent rise in stromal cell death was mostly due to the apoptosis of capillary endothelial cells (Fig. 4B). CD31 labelling of capillaries also showed that, despite increased endothelial cell apoptosis, the ratio of capillary density to myofibre density was not significantly ($P > 0.6$) altered by ageing.

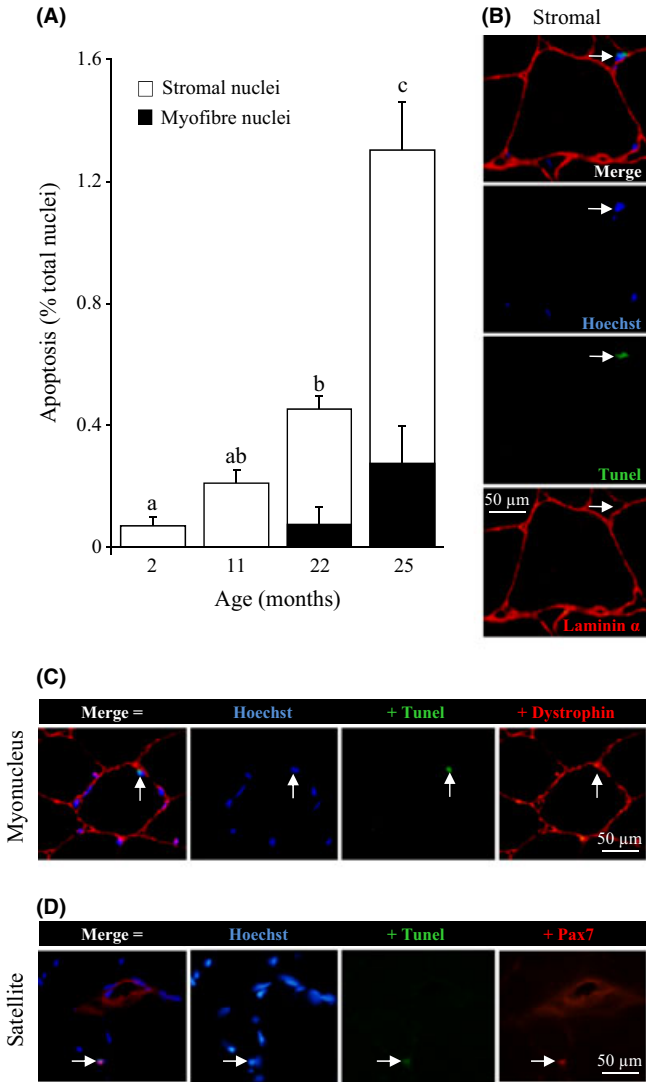

Fig. 3 Increased apoptosis in old mouse muscle. (A) Proportion of apoptotic nuclei in 2–25 months mice *gastrocnemius* muscle as revealed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL). Colabelling for laminin 2α distinguishes apoptosis in myofibers (myonucleus and satellite, black bars) and stromal cells (white bars). Values are means ± SEM ($n = 4$), and different letters indicate significant difference ($P < 0.05$) between ages. (B) Representative images of a 25 months muscle cross section labelled with Hoechst 33258 (blue), TUNEL (green) and anti-laminin 2α (red); the arrow indicates an apoptotic stromal cell nucleus. (C) Apoptotic myonucleus (arrow) in 25 months muscle cross section colabelled for Hoechst 33258 (nuclei, blue), apoptosis (TUNEL, green) and dystrophin (plasma membrane, red). (D) Apoptotic satellite cell (arrow) in 25 months muscle cross section co-labelled for Hoechst 33258 (nuclei, blue), apoptosis (TUNEL, green) and paired box protein-7 (Pax7) (satellite cell, red).

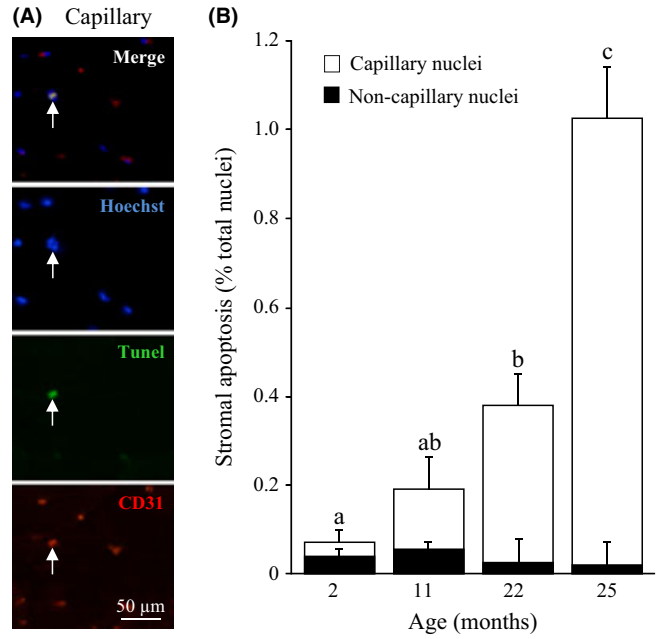

Fig. 4 Apoptosis in capillary endothelial cells. (A) Apoptotic capillary endothelial cell (arrow) in 25 months muscle cross section co-labelled for Hoechst 33258 (nuclei, blue), apoptosis (TUNEL, green) and CD31 (capillary endothelial cell, red). (B) Age-dependent increase in stromal and capillary endothelial cell apoptosis (white bars). Few non-capillary stromal apoptotic cells (black bars) remain to be identified. Values are means ± SEM ($n = 4$), and different letters indicate significant difference ($P < 0.05$) between ages.

Age-dependent modifications in extracellular matrix structure

Alterations in myofibre size and morphology, together with apoptosis in stromal cells, were in agreement with the profound remodelling of ECM that we observed during ageing (Fig. 5A). To characterize ECM modifications, image analyses were performed with muscle cross sections stained with Sirius red which labels major ECM components, type I and type III collagens (Tullberg-Reinert & Jundt, 1999). The total area (Fig. 5B) and total length (Fig. 5C) of ECM skeleton in GM cross sections both showed similar U-shaped evolutions ($P < 0.02$) with a minimum value at 11 months, and a maximum at 25 months. The muscle content of collagen hydroxyproline also increased in 25 months muscle (Fig. 5D, $P < 0.02$), which confirmed the emergence of ECM fibrosis in the old muscle. Additionally, old GM revealed enrichment in non-reducible hydroxylysylpyridinoline collagen cross-linking (Fig. 5E, $P < 0.01$) implicating an increased muscle stiffness.

The atrophy and reduced cross-sectional area of the old myofibres might be expected to be associated with an increased ramification of ECM embedding myofibres. Therefore, we further analysed ECM segments between pair of myofibres. While the mean length of ECM segments decreased (Fig. 5F, $P < 0.03$), the density of ECM connection points (where ≥ 3 segments interact) increased during ageing (Fig. 5G, $P < 0.02$), which confirmed an increased ramification of ECM.

Age-dependent modifications in extracellular matrix components

To further assess changes in ECM at the molecular level, several ECM components were investigated. Type VI collagen and tenascin-X are known to be localized both in the endomysium and the perimysium, while type IV collagen, laminin 2α and perlecan are exclusively located in the endomysial basement membrane (Voermans *et al.*, 2008). Semi-quantitative immunohistochemistry of type IV and VI collagens and of laminin 2α did not reveal significant changes during ageing (data not shown). However, age-dependent modifications were observed for tenascin-X and perlecan immunolabelling in the endomysium, but with distinct timings. Tenascin-X, which determines the mechanical properties

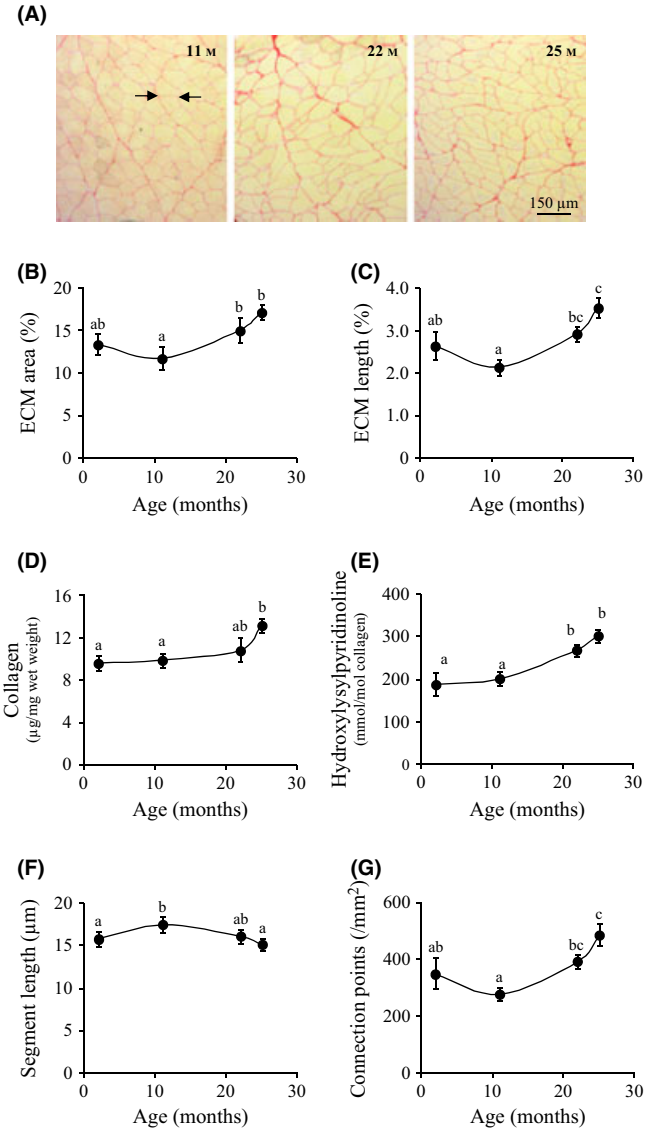

Fig. 5 Age-dependent variations in extracellular matrix (ECM) characteristics. (A) Images of *gastrocnemius* muscle cross sections stained with Sirius red at 11, 22 and 25 months. Myofibres and ECM are yellow and red, respectively. A connection point (arrow) is defined as the interaction between ≥ 3 myofibres, and each ECM segment is located between two connection points. Age-related variations in (B) ECM total area (% of total area), (C) ECM total length (% of total area), (D) collagen content, (E) hydroxylysylpyridinoline content, (F) ECM mean segment length and (G) number of connection points. All values in the graphs are means ± SEM (vertical bars) for $n = 4$ mice per age, and different letters indicate significant difference ($P < 0.05$) between ages.

of collagen (Margaron *et al.*, 2010), increased between 2 and 11 months, and remained stable thereafter (Fig. 6A,B, $P < 0.03$). In contrast, perlecan immunolabelling progressively reached a maximum at 22 months and decreased at 25 months (Fig. 6C,D, $P < 0.05$).

Previous studies on capillaries reported that apoptosis of endothelial cells is associated with a limited proteolysis of perlecan domain V (endorepellin) and with the production of peptides (called LG1–LG2 and LG3) that might be important for fibrosis of ECM (Laplante *et al.*, 2005). Because we observed that ageing in mice skeletal muscle was associated with (i) enhanced apoptosis of capillary endothelial cells, (ii) altered expression of perlecan and (iii) increased ECM fibrosis, we hypothesized that endorepellin fragments might be produced in old muscles. Western blots of total muscle extracts with a specific antibody to endorepellin identified a 63-kDa fragment (Fig. 6E) that corresponds to endorepellin LG1–LG2 and revealed that GM ageing was associated with a sharp increase in this endorepellin 63 kDa fragment (Fig. 6F).

Discussion

Age-dependent atrophy of skeletal muscle is associated with profound alterations in myofibres and with remodelling of the connective tissue embedding myofibres. The diminution in myofibre cross-sectional area and nuclear domain, together with myofibre-type grouping that we observed in the GM of old mice, are in agreement with the previous studies on human (Andersen, 2003) and rat (Yarovaya *et al.*, 2002) skeletal muscles. Image analyses were further used in the present study to demonstrate enhanced angularity, a notion previously mentioned but not specifically quantified in old muscle (Andersen, 2003).

The increased stiffness and reduced function of the old muscle are also associated with ECM fibrosis and increased collagen concentration and cross-linking (Kragstrup *et al.*, 2011). Herein we further extend this notion using image analyses to provide evidence that fibrosis is associated with thickening of ECM endomysium and an increased ramification of the ECM, which is required for myofibre atrophy.

Myonuclear apoptosis is a rare event in the old muscle

Age-dependent alterations in myofibre and ECM morphology were associated with modifications in the different populations of nuclei belonging to myofibres and to muscle connective tissue. Myofibre myonuclei presented a reduced myonuclear domain and an increased centralization in old skeletal muscle. Centralized myonuclei are recognized markers of regenerating myofibres (Yablonka-Reuveni & Anderson, 2006). However, in the old atrophying muscle, centralized myonuclei could also result from myofibre denervation and myofibre branching (Valdez *et al.*, 2010). In addition, alterations in the microtubule network during ageing (Piec *et al.*, 2005) might also cause changes in the distribution of the myonuclei (Bruusgaard *et al.*, 2006). Previous investigations about cell death in old skeletal muscle suggested that apoptotic nuclei were mostly myonuclei, although the identification of the nuclei has not been unambiguous. Our current study in mice indicates that myonuclear apoptosis could not be detected and thus is a rare event in young and adult GM. Nonetheless, apoptotic myonuclei did appear during ageing, although these did not account for more than 20% of total apoptosis in the old mouse GM. Myonuclear apoptosis is therefore a rare event also in old mouse muscle (0.8% of total myonuclei), which may nonetheless be important for the functionality of myofibres (Marzetti *et al.*, 2010).

Increased apoptosis of satellite cells in the old muscle

Age-related muscle loss is believed to partly result from the diminishing ability of muscle to repair itself (Shavlakadze *et al.*, 2010). Satellite cells function as myogenic progenitors in adult muscles, and an age-linked decline in satellite cell regenerative potential may limit repair of old muscles. The capacity of satellite cells to support muscle maintenance depends on their abundance, on their myogenic potential and on their local environment. In GM, the major decrease in satellite cell abundance occurs by 11 months of age during maturation, that is, before ageing. Previous studies performed in C57BL6 mice similarly showed that in

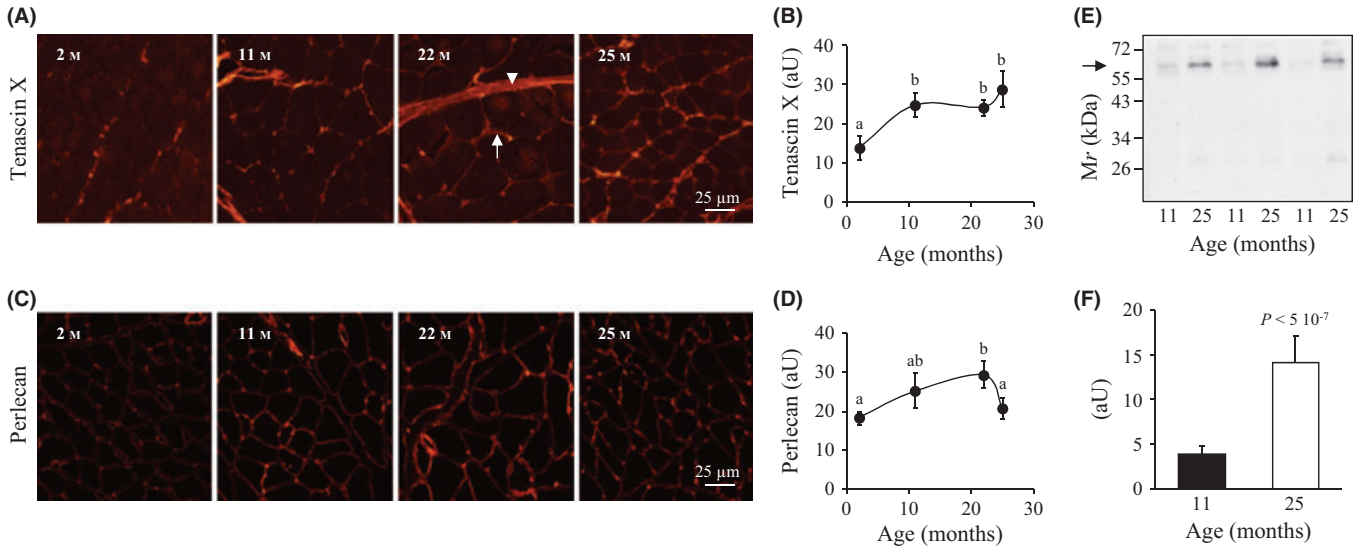

Fig. 6 Age-related variations in tenascin-X and perlecan. Representative images of *gastrocnemius* muscle cross sections from 2- to 25-month-old mice immunolabelled with (A) anti-tenascin-X and (C) anti-perlecan; perimysium (arrow head) and endomysium (solid arrow) are indicated. Semi-quantitative estimation of (B) tenascin-X and (D) perlecan immunolabelling in muscles during ageing. (E) Whole muscle lysates from 11 and 25 months muscles were analysed by Western blotting using a monoclonal anti-endorepellin antibody; the arrow indicates the LG1-LG2 endorepellin fragment; (F) corresponding densitometry analysis of the blot showing the increase in LG1-LG2 peptide.

extensor digitorum longus muscle, satellite cell number decreases before 1 year of age (Shefer *et al.*, 2006). While satellite cell number remained at a low level in old muscles, we also provide evidence that apoptosis occurs for a significant proportion of old satellite cells. This is in agreement with indications that satellite cells derived from old rat muscle demonstrate an increased susceptibility to apoptosis *in vitro* (Jejurikar *et al.*, 2006). Previous studies have demonstrated increased apoptotic satellite cells in response to exercise (Podhorska-Okolow *et al.*, 1998) and denervation (Bruusgaard & Gundersen, 2008) in limb muscles, but the importance of ageing for satellite cell apoptosis was only acknowledged in thyroarytenoid muscle for laryngeal function (Malmgren *et al.*, 2001). Our observations in GM now indicate that satellite cell apoptosis is also associated with age-related loss of limb muscles. Such apoptosis likely contributes to the decline of satellite cells with age, but may not result in major functional deficit. Previous studies indeed emphasized that the few satellite cells that survive the effects of ageing retain a full potential for muscle regeneration (Collins *et al.*, 2007; Shavlakadze *et al.*, 2010; Alsharidah *et al.*, 2013). In old mice muscle, the reduced number of Pax^+ satellite cells is partly counterbalanced by non-myogenic cells located in the same niche, but the function and origin of those cells is not clarified (Collins *et al.*, 2007).

Capillary endothelial cells contribute to apoptosis in the adult muscle

The present study further emphasizes that besides myofibre myonuclei and satellite cells, stromal cells of ECM account for a significant part of total nuclei in mice GM. Similar estimations of stromal cell proportion were provided for rat (Schmalbruch & Hellhammer, 1977) and mice (Murray & Robbins, 1982) skeletal muscles. Although stromal cells comprise 30–40% of the total nuclei in adult rodent skeletal muscle, their contribution to apoptosis nevertheless has been largely neglected. Apoptotic nuclei are rare in normal adult mice GM, and in accordance with the previous studies in adult rat muscle (Allen *et al.*, 1997), our triple labelling (lamina, TUNEL, Hoechst) showed that the majority of apoptotic nuclei were located in ECM in the adult muscle. Our study now identifies these rare apoptotic cells in adult muscle as $CD31^+$ capillary endothelial cells.

Capillary endothelial cells contribute to apoptosis in the old muscle

Previous studies in different models of atrophying muscles reported that apoptosis is not confined to myofibres, as apoptosis occurs in stromal cells in response to underweighting (Allen *et al.*, 1997), hypertension (Gobé *et al.*, 1997), heart failure (Vescovo *et al.*, 1998) and in dystrophic muscles (Sandri *et al.*, 1998). However, the implication of stromal cells in muscle apoptosis was not previously mentioned during the ageing process. While ageing was associated with a detectable but limited rise of apoptosis in myonuclei, we provide evidences that apoptotic cells in old GM are mostly located in ECM. Moreover, our data identify $CD31^+$ capillary endothelial cells as the major apoptotic cells in sarcopenic muscle. Apoptosis was observed in muscle capillaries in response to high salt intake in rats (de Resende *et al.*, 2006), but was not previously reported in capillaries of aged skeletal muscle.

Ageing predisposes to a progressive impairment of the vasculature, and several studies reported the deleterious impact of age on endothelial functions in the peripheral microcirculation (Herrera *et al.*, 2010; Virdis *et al.*, 2010). One mechanism for this endothelial dysfunction is the increased density of apoptotic endothelial cells (Asai *et al.*, 2000). During vascular remodelling, apoptosis in endothelial cells triggers the release of fragments of endorepellin (perlecan domain V) (Laplante *et al.*, 2005). Endorepellin contains three laminin-like globular domains (LG1 to LG3). While LG3 relatively freely diffuses and can be detected in many body fluids, LG1–LG2 binds specifically to major basement membrane constituents (Whitelock *et al.*, 2008; Iozzo *et al.*, 2009). In agreement with these observations, we report that apoptosis of capillary endothelial cells in the old skeletal muscle is coincident with the production of LG1–LG2 endorepellin fragment. However, we cannot specify whether muscle cells, other than endothelial cells, also contribute to the production of

LG1–LG2 fragment. Matrix proteases, such as plasmin, collagenase and stromelysin (Whitelock *et al.*, 2008), but also oxidative damage (Rees *et al.*, 2010), could be involved in partial proteolysis of perlecan, while bone morphogenetic protein-1 (BMP-1/Tolloid) or cathepsin L could cleave LG3 from endorepellin/perlecan (Whitelock *et al.*, 2008).

Our study indicates that apoptosis of capillary endothelial cells accounts for more than 75% of apoptosis in the old mouse muscle. Regeneration mechanisms, such as division or hyperplasia of adjacent endothelial cells, or joint of circulating endothelial progenitor cells (Herrera *et al.*, 2010), are also likely involved to maintain capillary to myofibre ratios in the old muscle. Overall, these phenomena may be of importance given the fundamental role of endothelial cells in the regulation of vascular homeostasis. Of note, blood vessels and satellite cells could be in close vicinity, and satellite cells may influence angiogenesis, and reciprocally, endothelial cells may enhance satellite cell growth (Christov *et al.*, 2007). Strikingly, these two cell populations were the most affected by apoptosis in sarcopenic muscle of old mice. If circulating factors trigger cell damage, capillary endothelial cells and juxtavascular satellite cells may be subjected to harmful stimuli to a higher degree than myofibres. In turn, dysfunction in endothelial cell turnover could disturb the integrity of the endothelial monolayer and produce profound alterations in the delivery of nutrients and oxygen and in the removal of toxic metabolic products.

Experimental procedures

Animals, muscle samples

C57BL6 male mice (Jackson Laboratory, Singapore, Singapore) were from the Laboratory Animal Centre of the National University of Singapore and raised in the veterinarian-staffed Laboratory Animal Facility at Nanyang Technological University (NTU) following the procedure of the Institutional Animal Care-and-Use Committee. Mice were housed in a temperature (22 ± 1 °C)- and humidity (50–70%)-controlled facility, with a 12:12 h light–dark cycle, and food (SAFE, Singapore, Singapore) and water were provided *ad libitum*. Mice were either 2, 11, 22 or 25 months of age, corresponding to young adult, mature adult, early old or advanced old mice, respectively. Mice were killed by cervical dislocation after CO_2 anaesthesia and weighted, and GM were rapidly removed from both hind limbs and weighted. Samples from the mid-belly of the lateral and medial heads of GM were used. Muscle samples were either snap-frozen in liquid nitrogen or for histological analyses mounted on cork board and frozen in isopentane cooled on liquid nitrogen. Four serial cross sections (10 μm thickness) were collected at 100-μm intervals throughout the entire sample using a cryostat (Microm, Francheville, France) at −25 °C.

Muscle extracellular matrix and myofibres structure

Muscle ECM and myofibre characteristics were studied on cross sections stained with Sirius red (Tullberg-Reinert & Jundt, 1999). At least four cross sections (each corresponding to 100–250 myofibres) were analysed per mouse and for four to five mice per age. Sirius red stains ECM red and myofibres yellow, and provides important contrast between myofibres and ECM, well suitable for image analysis.

Images were captured with a DP-72 camera coupled to a BX-51 microscope (Olympus, Rungis, France) at a resolution of 0.32 or 0.16 μm per pixel. Five colour images per mouse were acquired under identical conditions (exposure time, white balance) in bright fields. Sirius red images were processed through a homemade visual basic program developed under VISILOG 6.7 software (Noesis, Gif sur Yvette, France). First, inverted optical density greyscale images were obtained from the green component of the colour images. Second, thresholding was carried out followed by skeletonization. Therefore, in the resulting binary image, ECM area and length, ECM segment length and number of connection points were derived out. A connection point was defined as the interaction between ≥ 3 myofibres and a segment as ECM link between two connection points. Reconstruction of binarized images was carried out and the total number of myofibres and characteristics of each myofibre (number of neighbours, cross-sectional area) were recorded.

Oxidative metabolism of myofibre was assayed by cytochrome c oxidase (Cox) histochemistry (Bio-Optica, Milan, Italy). Slow-twitch oxidative myofibres were Cox positive (dark brown), while fast-twitch glycolytic myofibres were Cox negative.

Myonuclei

Total muscle nuclei were visualized on 10 μm cross sections stained with Hoechst 33258 (Sigma, L'Isle d'Abeau Chesnes, France). To distinguish myofibre nuclei (myonuclei), plasma membrane was labelled for dystrophin. After fixation with 4% paraformaldehyde for 10 min and antigenic site saturation (5% BSA in PBS) for 30 min, sections were incubated with primary rabbit anti-dystrophin (1:500; Abcam, Paris, France) for 1 h, rinsed with PBS three times and incubated with the secondary antibody conjugated to DyLight 488 (1:400; Interchim, Montluçon, France) for 45 min in the dark. Muscle sections were then washed twice with PBS, incubated 1 min with PBS containing 2 μg mL^{-1} Hoechst 33258 and mounted with Gel Mount (Sigma).

For all immunofluorescence analyses, images were captured with a DP-72 camera coupled to a BX-51 microscope at a resolution of 0.32 or 0.16 μm per pixel. Ten images per mouse, for four to five mice per age, were captured under optimal condition (exposure time) through adequate filters: blue (excitation at 345 nm, emission at 450–490 nm) for Hoechst 33258, green (excitation at 470 nm, emission at 510–560 nm) for DyLight 488 and red (excitation at 530 nm, emission at 575–650 nm) for DyLight 549. Negative controls were performed by omitting either the primary or secondary antibody on serial sections.

Satellite cell nuclei

Satellite cell nuclei were identified by costaining with anti-Pax7 (paired box protein 7) and Hoechst 33258. Anti-Pax7 labels both quiescent and activated satellite cells (Péault *et al.*, 2007). After saturation (5% BSA in PBS for 30 min), cross sections were incubated successively with 10% unconjugated AffiniPure Fab fragment anti-mouse IgG (H+L) (Interchim) for 1 h, with mouse monoclonal anti-Pax7 (1:50; Hybridoma Bank, Iowa City, IA, USA) for 1 h and with secondary antibody conjugated to DyLight 549 (1:400; Interchim) for 45 min. Hoechst 33258 staining was used to verify that Pax7 labelling corresponds to nuclei.

Stromal cell nuclei

To distinguish ECM stromal nuclei, myofibre basal lamina was labelled for laminin 2α. After fixation with cold acetone for 10 min and antigenic site saturation (5% BSA in PBS) for 30 min, sections were incubated with primary rat anti-laminin 2α (1:200; Abcam) for 1 h, rinsed with PBS three times and incubated with the secondary antibody conjugated to DyLight 549 (1:400) for 45 min in the dark. Muscle sections were then washed twice with PBS, incubated 1 min with PBS containing 2 μg mL^{-1} Hoechst 33258 and mounted with Gel Mount.

Apoptotic nuclei

Detection of apoptotic nuclei was performed using a TUNEL (Terminal deoxynucleotidyl transferase fluorescein-dUTP nick end-labelling) fluorescent detection kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. TUNEL assay might also detect naturally occurring single-stranded DNA breaks, although this has mostly been reported in differentiating muscle cells (Larsen *et al.*, 2010). This phenomenon is thus unlikely to be predominant in the old atrophying muscle, as myofibres not subjected to hypertrophic stimulus are refractory to satellite cell fusion. Apoptotic nuclei were counted and localized for approximately 1300 myofibres per mouse and for four mice per age. For all groups, positive controls were carried out on serial sections after prior incubation with 0.12 μg μL^{-1} DNAse I, while negative controls used labelling solution instead of TUNEL solutions. Costaining of apoptotic nuclei with anti-laminin 2α (1:200), anti-dystrophin (1:500) or anti-CD31 (1:100; Abcam) was performed after the TUNEL assay. Briefly, sections were rinsed with PBS for 30 min, saturated by 5% BSA in PBS for another 30 min, incubated with the primary antibody for 1 h and then with the secondary antibody conjugated to DyLight 549 for 45 min. Hoechst 33258 was also used to avoid false-positive TUNEL. Similar protocols were applied for costaining of apoptotic nuclei with Pax7, except that cross sections were pre-incubated overnight at 4 °C with unconjugated AffiniPure Fab fragment (1:20) before the incubation with mouse primary antibody.

Extracellular matrix composition

Expression of several ECM components was studied by double indirect immunostaining on 10 μm thick sections for four animals per age. Guinea pig polyclonal anti-tenascin-X (1:100) was previously described (Margaron *et al.*, 2010). Rabbit polyclonal antibodies to type IV collagen and type VI collagen (both 1:40; Novotec, Lyon, France), rat monoclonal to perlecan (1:1; Abcam), and guinea pig anti-tenascin-X were applied after saturating (5% BSA in PBS). Rat anti-laminin 2α was applied after acetone fixation and saturating. Incubations with corresponding DyLight 488- or 549-conjugated secondary antibodies (1:400) were for 45 min at room temperature**.** For each image, pixel intensity was randomly collected for 10 endomysial regions. Images were processed with ImageJ version 1.42q (NIH, Bethesda, MD, USA).

Collagen and cross-link content

To estimate total collagen, frozen muscle powder (100 mg) was hydrolysed in 2 mL HCl 6 N overnight at 105 °C, incubated with activated charcoal (Norit A; Sigma) and diluted with four vol H_2O. Hydroxyproline content was determined according to the procedure of Woessner (1961), and optical density was measured at 557 nm. Collagen content was calculated assuming that 14% amino acid in collagen is hydroxyproline.

Hydroxylysylpyridinoline cross-links were measured on the same hydrolysate. After 5 min centrifugation at 16 000 *g* and 4 °C, 300 μL of supernatants was added to 300 μL NaOH 6 N and 300 μL Tris 1 M. Final pH was adjusted between 6.5 and 7.5. Cross-links were determined in duplicate using the enzyme-linked immunoassay Metra Pyd EIA kit (Teco Medical, Paris, France) according to the manufacturer's instructions. Cross-link concentration was expressed as millimoles of hydroxylysylpyridinoline per mole of collagen, assuming the molecular weight of collagen is 300 000.

Immunoblotting

Muscles were lysed in ice-cold buffer containing 8.3 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT) and were clarified at 10 000 *g* for 30 min. Aliquots (20 μg protein) were resolved by SDS-PAGE (12%), electrotransferred to Hybond-P PVDF membranes (Dutscher, Brumath, France) and probed with a mouse monoclonal anti-endorepellin (A74; Abcam) diluted (1:200) with 5% milk in 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20. Primary antibody was resolved using a peroxidase-conjugated secondary antibody (1:5000) and ECL Plus system (GE Healthcare, Velizy-Villacoublay, France). Signals recorded were quantified using Quantity One software (Bio-Rad, Marnes La Coquette, France) and normalized against the amount of proteins (determined following Ponceau Red staining) to correct for uneven loading.

Statistical analysis

All data are expressed as means ± SEM. Statistical analyses of each dependent variable were carried out using one-way ANOVA. When the data did not satisfy the normality criterion, the Kruskal–Wallis method was used. Multiple comparisons of the honestly significant differences were assessed by Fisher's test. The P value < 0.05 was used as the basis for the conclusion of significant difference.

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Author contributions

DB, AL, and KLG conceived and designed the experiments. HW, AL, CCG, and MG performed the experiments. DB, HW, BM, and AL analysed the data. CL, LC, DT, CP, and DA contributed reagents/materials/analysis tools. DB, HW, and AL wrote the paper. DA, KL, and KLG revised the manuscript.

Conflict of interest

None declared.

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