Chikungunya virus infection involved monocytes and during chronic phase of the disease persisted in tissue macrophages

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Workshop 1: Phagocyte Biology: From basic science to pathological aspects

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Immune cell recruitment in infection and sterile inflammation

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Recruitment of various leukocytes is the key feature of any immune response. However, the response may vary among different leukocytes, among different organs and perhaps even among various insults for the same cell type in the same organ. In this presentation, I will highlight how the immune response can differ between tissues such as muscle and liver. While selectins and integrins are critical for cell recruitment into muscle, in liver there is often but not always much less role for integrins and almost no role for selectins. In liver CD44 becomes a dominant molecule for neutrophil recruitment in response to infectious agents. Surprisingly, despite very similar patterns of neutrophil recruitment in sterile injury, CD44 plays no discernable role and the neutrophils use more traditional mechanisms of recruitment including the integrin Mac-1. Surprisingly, the neutrophil arrives at the sterile injury site due to activation of its fMLP receptors, molecules thought to be important for chemotactic responses to infections. Clearly despite very similar find me signals between infection and sterile inflammation, the neutrophil uses different adhesive mechanisms of recruitment.

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Role of P-Rex and Vav family GEFs in thioglycollate induced neutrophil recruitment

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Background: Neutrophils constitute the first line of cellular defence against bacteria and fungal invasion. They respond rapidly to chemoattractants and migrate to the site of infection where they ingest the invading microbes, kill them by releasing granule contents and producing reactive oxygen species (ROS). The small G protein Rac regulates cellular motility and plays important roles in neutrophil adhesion, spreading and chemotaxis. Guanine nucleotide exchange factors (GEFs) of the P-Rex and Vav family activate Rac and are involved in the regulation of neutrophil functions. The aim of this project is to study the role of these GEFs in neutrophil migration to the site of inflammation.
Materials and methods: Mice deficient in either the P-Rex family (P-Rex1$^{-/-}$, P-Rex1$^{-/-}$P-Rex2 $^{-/-}$) or the Vav family (Vav1$^{-/-}$/Vav2$^{-/-}$/Vav3$^{-/-}$) or a combination of P-Rex and Vav family GEFs (P-Rex1$^{-/-}$/Vav1$^{-/-}$ and P-Rex1$^{-/-}$/Vav3$^{-/-}$) were used. The ability of these animals to recruit neutrophils to the peritoneal cavity upon thioglycollate challenge was studied.
Results: Mice lacking the member of both P-Rex and Vav family GEFs were less capable of recruiting neutrophils to the site of inflammation than animals deficient in either the entire P-Rex family or the entire Vav family. Mice deficient in both P-Rex1 and Vav3 showed the biggest defect in neutrophil recruitment. This implies that neutrophil migration *in vivo* requires the presence of members from both families of Rac-GEFs.
Conclusions: P-Rex and Vav family together regulate neutrophil recruitment to the site of inflammation.

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Ontogeny of neutrophil recruitment in the murine and human fetus

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Background: Life-threatening bacterial infections are still a major cause of neonatal morbidity and mortality in premature infants, but rare in mature neonates. One mechanism responsible for the high risk of sepsis in prematures might be an attenuation of neutrophil recruitment. To test this hypothesis, we investigated neutrophil recruitment in mice and human fetuses throughout gestation.
Material and methods: Using a novel murine intravital microscopy model, we studied neutrophil recruitment during fetal development *in-vivo*. In addition, we performed microflow chamber experiments to examine rolling and adhesion of neutrophils isolated from murine fetuses or from umbilical cord blood of premature and mature newborns. To investigate the adhesive properties of fetal endothelial cells (EC), we measured recruitment of adult neutrophils on LPS-treated HUVEC from premature and mature neonates.
Results: Neutrophil recruitment in inflamed yolk sac vessels was almost absent in E13–E14 embryos, but steadily increased at later time points. Likewise, rolling and adhesion of murine and human neutrophils in flow chambers correlated significantly with gestational age and could hardly be observed at midgestation. Interestingly, rolling velocity was also significantly reduced in premature neonates. The adhesive properties of neonatal EC were equally dependent on gestational age with HUVEC from extreme prematures showing the lowest ability to recruit adult neutrophils. A follow-up experiment on extreme prematures provided evidence that maturation of PMN recruitment is not altered by extrauterine factors as compared to intrauterine development.
Conclusion: Neutrophil recruitment is ontogenetically regulated during human and murine fetal development involving both, neutrophil and EC functions. In this context, the inability of very preterm infants to sufficiently recruit neutrophils is

likely to contribute to their increased susceptibility to life-threatening infections.

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The receptor for advanced glycation endproducts RAGE controls P-selectin-dependent leukocyte rolling *in vivo*

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Background: The receptor for advanced glycation endproducts RAGE has been implicated to contribute to the inflammatory response in a variety of acute and chronic diseases. This has been mostly attributed to its ability to mediate signaling via NK-κB leading to the upregulation of several proinflammatory cytokines and adhesion molecules.
Material and methods: Using intravital microscopy and flow chamber assays, we studied leukocyte rolling in the absence of RAGE.
Results: We show in trauma-stimulated and TNF-α-stimulated cremaster muscle venules of RAGE deficient mice that P-selectin-dependent rolling is significantly reduced compared to wild type mice. To clarify whether the reduction in P-selectin dependent rolling is caused by defective P-selectin ligand activity on leukocytes or due to a functional attenuation of P-selectin activity on the endothelium, we generated bone marrow chimeric mice ($RAGE^{-/-}$ into WT; WT into $RAGE^{-/-}$). We found normal P-selectin dependent rolling in inflamed cremaster muscle venules of $RAGE^{-/-}$ into WT mice while P-selectin dependent rolling was significantly reduced in WT into $RAGE^{-/-}$ mice suggesting that endothelial RAGE controls P-selectin dependent rolling *in vivo*. Additional experiments using *in vitro* and *ex vivo* flow chamber assays, demonstrated normal P-selectin ligand function on leukocytes and also excluded any soluble plasma factors for the observed reduction in P-selectin dependent leukocyte rolling.
Conclusion: These findings provide strong evidence that endothelium expressed RAGE controls P-selectin dependent rolling during acute inflammation *in vivo*.

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Calpain-1: investigating its role in murine neutrophils

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Background: Extravasation and phagocytosis are important processes which involves complex signals within the neutrophils that may include the activation of the cytosolic Ca^{2+} activated protease, calpain-1. This work investigates the role of calpain-1 in regulating Ca^{2+} activated neutrophils functions such as transendothelial migration, phagocytosis process and spreading using neutrophils from a calpain-1 knock-out (KO) mouse.
Materials and methods: By using intracellular sperm injection (ICSI) from heterozygous mice generated (EMMA), and a genotype-based breeding programme, a colony of homozygous calpain-1 KO mice has been generated in Cardiff. Neutrophils were isolated from the blood of the mice and studies were conducted comparing the normal wild-type and homozygous calpain-1 KO cells.
Results: Neutrophils isolated from the homozygous calpain-1 KO mice circulation had a significant defect in the ability to migrate across ICAM-1 expressing endothelial cell monolayers in *in vitro* transmigration assays. This effect was only evident with TNF-treated endothelial cells, pointing to a specific defect in the β2 integrin/ICAM-1 signalling process. As calpain-1-deficient cells signalled cytosolic Ca^{2+} in response to β2 integrin engagement (C3bi-opsonised zymosan), the defect was downstream of Ca^{2+}. SEM imaging of homozygous calpain-1 KO neutrophils revealed that their surface is also less wrinkled and may therefore have a smaller 'membrane reservoir'.
Conclusions: These data were consistent with calpain-1 activation by Ca^{2+} being an important event in trans-endothelial migration. Differences in morphological surface and spreading area of the cells could also be responsible for the transmigration effects.

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Differentiation and functions of the 'mononuclear phagocytes'

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Monocytes and macrophages are critical effectors and regulators of inflammation and the innate immune response, whereas dendritic cells initiate and regulate adaptive immune responses, and are central to the development of immunologic memory and tolerance.

Recent *in vivo* experimental approaches in the mouse have unveiled new aspects of the developmental and lineage relationships among these cell populations. Monocytes, and a number of macrophages and dendritic cells subsets share a common bone marrow progenitor, the MDP (Macrophage and DC progenitor), which express receptors for SCF, M-CSF and FLT3-L, and the chemokine receptor CX3CR1. However, distinct subsets of dendritic cells and macrophages are generated via separate differentiation pathways, which are beginning to be elucidated. In addition, some resident macrophages and DCs appear to differentiate during embryogenesis, together with their dedicated tissues, and may be independent from the bone-marrow for their renewal and homeostasis in adults.

Blood monocytes themselves consist in several functional subsets. A population of Gr1 + 'inflammatory' monocytes and their human counterpart CD14 + monocytes recognise bacteria, fungi and viruses, extravasate to the site of infection and inflammation and give rise to 'inflammatory' macrophages and DCs. A distinct monocytic subset, the murine Gr1- monocytes and their human equivalent $CD14^{dim}$ monocytes, adhere to and patrol endothelium of blood vessels and specialize in innate immune response to viruses and nucleic acids via a unique MyD88-MEK pathway, and may contribute to the homeostasis of endothellium. Functions of monocytes and the molecular mechanisms involved, and genetic and epigenetic variations that may cause human inflammatory diseases are actively investigated in the laboratory.

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Characterization of human macrophage 3D migration: cellular mechanisms and molecular determinants

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Background: Tissue infiltration of macrophages, although critical for innate immunity, is also involved in pathologies, such as chronic-inflammation and cancer. *In vivo*, macrophages migrate mostly in a constrained three-dimensional (3D) environment. We have recently shown that, depending on the extracellular matrix (ECM) architecture, human macrophages are able to migrate in 3D using either the protease-independent amoeboid migration mode (like other leukocytes) or the protease-dependent mesenchymal migration mode.

Our aims were next (i) to better characterize macrophage mesenchymal migration, (ii) to determine whether the same macrophage was able to perform both 3D migration modes, and (iii) to define molecular determinants of 3D-migration by transcriptomic analysis.

Materials and methods: Human macrophages derived from blood monocytes (MDMs) are used in 3D-migration assays into ECM of different architectures and cell spheroids.

Results: 1. Characterization of macrophage mesenchymal migration: MDM migration through ECM is MMP-independent but requires lysosomal proteases, whereas MDM migration through cell spheroids is MMP dependent. In addition, we show that this migration mode involves three cellular processes: (i) matrix degradation through the formation of 3D podosomes; (ii) matrix compaction and (iii) matrix internalization.

2. By using composite matrices, we show that a single macrophage is able to switch from amoeboid to mesenchymal migration and vice versa.

3. Identification of molecular determinants of MDM 3D migration by transcriptomic analysis: Gene expression profiles were compared between 2D- and 3D-MDMs migration and between 3D-amoeboid vs. 3D-mesenchymal migration. Analysis of transcriptomic signatures are currently under analysis.

Conclusions: Characterization of macrophage mesenchymal migration will lead to identification of potential inhibitors specific for macrophage tissue infiltration.

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Adhesive and effector functions of neutrophils after they migrate through endothelium into matrix *in vitro*

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Background: Relatively little is known about regulation of neutrophil function after migration into tissue. We used an *in vitro* culture model to investigate adhesive and functional changes.

Materials and methods: Neutrophils were tracked microscopically as they migrated through TNF-stimulated endothelial cells (EC) into collagen gels, and were retrieved at desired times. Oxidant production, integrin expression and apoptosis were analysed by flow cytometry and real-time quantitative PCR.

Results: Neutrophil migration was dependent on dose of TNF. At higher dose (100 U mL^{-1}) neutrophils migrated in greater number and to a greater depth than lower dose (1 U mL^{-1}). Judged by nuclear morphology and activation of caspase 3, apoptosis was barely detectable in neutrophils retrieved after 24 h, and most remained viable also at 48 h. The retrieved neutrophils (24 h) oxidised dihydroxyrhodamine to a similar level as freshly-isolated neutrophils when stimulated by fMLP. However, we found the total mRNA extracted from the retrieved neutrophils (24 h) was about half of that from fresh neutrophils. Nevertheless, β1- and β3-integrins, and ICAM-1 were markedly up-regulated in these cells, while β2-integrins were down-regulated, compared to fresh cells or neutrophils that were washed off the endothelial surface. Similar trends in gene expression were detected in neutrophils retrieved after 3 h.

Conclusions: Increasing concentration of TNF increased penetration of neutrophils into matrix as well the efficiency of crossing the endothelial monolayer. Migrated neutrophils survived much longer than cells cultured in dishes. Differential changes in expression of integrins after migration may represent changes in adhesive phenotype suited to movement through tissue. The functional correlates remained to be demonstrated in this respect, but perhaps surprisingly, the migrated cells do not appear to be primed for oxidant production.

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Macrophage migration requires expression and function of the tyrosine kinase Abl

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Background: The evidence that: (i) the tyrosine kinase Abl is associated with the integrin-bound Src-family kinases (SFKs) Hck and Fgr; (ii) expression of Hck and Fgr are indispensable for macrophage migration and lack of Hck and Fgr expression results in reduced tyrosine phopshorylation of Abl; (iii) inhibition of Abl by imatinib mesylate inhibits macrophage migration and polarization, led us to conclude that a cross-talk between SFKs and Abl is essential to regulate macrophage migration (Baruzzi et al. FEBS Lett. 584:15–21, 2010).

Materials and methods: A mixture of four siRNA (from Dharmacon) designed to silence Abl expression was transfected in bone marrow-derived macrophages (BMDM) by electroporation with the Amaxa nucleofector. After 72 h from transfection, BMDM were assayed for cell migration, polarization and podosome formation by standard assays.

Results: Delivery of siRNA in BMDM reduced by > 70% Abl expression without affecting Arg or actin expression. In Abl silenced BMDM, tyrosine phosphorylation of Abl substrates (CrkL and Stat5) was strongly reduced. Abl silenced BMDM displayed a marked alteration in cell migration in *in vitro* wound healing assays. Additionally, inhibition of Abl kinase activity or silencing of Abl expression resulted in reduction of podosome formation and cell polarization. Consistent with a cross-talk between SFKs and Abl, both the single Fgr and the double Fgr/Hck deficiency reduced podosome formation in BMDM.

Conclusion: Abl is an important determinant of polarization, migration and podosomes formation in macrophages.

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TLR2 expression is increased during *in vivo* transmigration of human neutrophils and results in a primed TLR response

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Background: Neutrophils are important in inflammation and arrive at injured sites after transmigration into the tissue from circulation. Tissue neutrophils have an altered surface flora of receptors, in part due to degranulation of intracellular receptor-storing granules, and are primed (hyper-responsive) to subsequent stimulation. Priming has been mostly investigated in relation to chemoattractants, the receptors for which are upregulated to the cell surface by degranulation. Less is known about the pathogen-associated molecular patterns (PAMP)-binding Toll-like receptors (TLRs) on *in vivo* transmigrated neutrophils, both regarding expression levels and whether transmigrated cells are functionally primed for PAMP activation in tissues.
Materials and methods: *In vivo* transmigrated neutrophils were extracted using a skin chamber technique and compared to neutrophils separated from peripheral blood from the same donors.
Results: *In vivo* transmigrated neutrophils displayed elevated levels of various receptors (e.g. complement receptors, chemoattractant receptors and cytokine receptors) including TLR2, as compared to peripheral blood neutrophils. *In vitro* priming of peripheral blood neutrophils also resulted in increased surface expression of TLR2, suggesting that TLR2 is stored in easily mobilized secretory organelles. Measuring IL-8 production, *in vivo* transmigrated neutrophils were hyper-responsive to various TLR2-binding PAMPs indicating that upregulation of TLR2 during transmigration renders neutrophils primed to PAMP activation in the tissues.
Conclusion: TLR2 upregulation from secretory organelles during *in vivo* transmigration could explain the increased expression of TLR2 and the hyper-responsiveness to TLR2 stimulation.

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Impact of midkine on the recruitment of neutrophils during acute inflammation

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Background: Acute inflammation is known to be compromised in the genetic absence of the cytokine midkine (MDK) in mice. Here we studied the functional impact of MDK for inflammatory activation of polymorphonuclear neutrophils (PMN) and endothelial cells as well as for PMN recruitment *in vivo*.
Materials and methods: Human PMN were isolated from peripheral blood of healthy adults. Murine PMN were obtained from the bone marrow of $MDK^{-/-}$ and $MDK^{+/+}$ mice. Human umbilical vein endothelial cells (HUVEC) were isolated from the umbilical cord. Cell surface expression of adhesion molecules was studied by antibody staining using flow cytometry. Adhesion was measured upon exposure of PMN to immobilized fibrinogen, a ligand of the β_2 integrin Mac-1 (CD11b/CD18). Leukocyte recruitment was investigated in cremaster muscle venules of $MDK^{-/-}$ and $MDK^{+/+}$ mice using intravital microscopy.
Results: In human PMN, soluble MDK did not induce up-regulation of Mac-1 (CD11b/CD18) expression or ROS production. Moreover, soluble MDK only slightly induced adhesion of PMN when compared to the proinflammatory cytokine TNFα. Similar results were obtained in murine PMN. In HUVEC, MDK was unable to up-regulate expression of ICAM-1 and VCAM-1 suggesting that soluble MDK does not induce an inflammatory activation of PMN and/or HUVEC. In contrast, immobilized MDK mediated strong PMN adhesion implying that it may exert its effects in a surface-bound form as adhesive substratum. Accordingly, leukocyte adhesion was significantly compromised in inflamed cremaster muscle venules of $MDK^{-/-}$ mice compared to $MDK^{+/+}$ animals. Similarly, perivascular PMN accumulation was significantly reduced. The adhesion defect was completely rescued upon intra-arterial injection of recombinant MDK.
Conclusion: These findings indicate a crucial role of MDK for adhesion and subsequent extravasation of PMN during acute inflammation.

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The role of neutrophils in hepatic insulin resistance induction after initiating high-fat feeding

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Background: Obesity is characterized by chronic inflammation of adipose tissue manifested by secretion of proinflammatory cytokines and macrophage infiltration. Obesity induces an insulin-resistant state in adipose tissue, liver, and muscle. Previous studies in our laboratory demonstrated that early (3 and 7 days) after initiating high-fat feeding of Male C57BL/6J mice, neutrophils transiently infiltrate the parenchyma of intra-abdominal adipose tissue. In addition, hepatic insulin resistance was detected as early as 3 days after initiating a high fat diet in the mice. In the present study, we aimed to define whether neutrophils recruitment into adipose tissue has a role in the induction of the hepatic insulin resistance.
Materials and methods: To prevent neutrophil recruitment into adipose tissue, mice received intraperitoneal injections of anti ICAM-1 antibodies or control nonspecific mouse IgG2b antibodies during the short course of high fat diet (HFD). After 3 days on diet, mice received insulin boost for 3 min and sacrificed. Periepididymal fat tissues and livers were collected and analyzed for the presence of neutrophils and insulin signaling, respectively.
Results: Intra-abdominal adipose tissue from HFD mice but not from mice on low fat diet (LFD) showed elevation in the presence of neutrophils (detected by myeloperoxidase expression) after 3 days on HFD diet. The treatment with ICAM-1 antibodies but not with the control antibodies significantly prevented the recruitment of neutrophils to the adipose tissue. The impairment in insulin signaling detected by immunoblotting of phospho-Akt in liver lysates of HFD mice was

prevented in HFD mice treated with anti ICAM-1 antibodies but not with control antibodies.
Conclusions: The treatment with anti ICAM-1 antibody prevented neutrophils recruitment into intra-abdominal adipose tissue and prevented impairment of hepatic insulin signaling early (3 days) after initiating high-fat feeding of Male C57BL/6J mice, suggesting that neutrophil recruitment process to adipose tissue play an important role in the induction of hepatic insulin resistance.

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Cytohesin-1 regulates fMLF-mediated activation of the β_2 integrin Mac-1 in neutrophils

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Background: The nucleotide exchange factor cytohesin-1 was previously reported to interact with the cytoplasmic domains of the integrin β chain common to all β_2 integrins such as LFA-1 and Mac-1. Though cytohesin-1 contributes to fMLF-induced signaling and functional responses in polymorphonuclear neutrophils (PMNs) through ARF6 activation, a role for cytohesin-1 in regulating Mac-1 activity has not been investigated.
Materials and methods: Using the cytohesin inhibitor secinH3, siRNA, Mac1 blocking mAbs, and mAb CBRM1/5 specific for the activation epitope of the αM subunit we have evaluated the role of cytohesin-1 in Mac-1-dependent adhesion to fibrinogen, migration and phagocytosis.
Results: We show here that cytohesin-1 restrains the activation of Mac-1 in PMNs or dibutyryl cAMP-differentiated PLB-985 cells. We found that the cytohesin-1 inhibitor secinH3 or siRNA increased cell adhesion to immobilized fibrinogen and fMLF-mediated conformational changes of Mac-1 monitored using mAb CBRM1/5 specific for the activation epitope of the αM subunit. In contrast, PLB-985 cells over-expressing cytohesin-1 showed little adhesion to fibrinogen. The use of secinH3 and siRNA also revealed that interference with cytohesin-1 signaling also enhanced phagocytosis of zymosan particles and chemotaxis towards fMLF in transwell migration assays. These increases in phagocytosis and chemotaxis in cells treated with secinH3 and cytohesin-1 siRNA were reversed by a Mac-1 blocking mAb. We provide evidence for increased polymerized cortical actin in cells treated with secinH3 and that altered signaling through cytohesin-1 increases cell surface expression of FPRL-1 and impairs the late calcium mobilization response elicited by fMLF.
Conclusions: The data provide evidence that stimulation with fMLF initiates a signaling cascade that restrains Mac-1 activation in PMNs. Such crosstalk between FPRL-1 and Mac-1 involves cytohesin-1.

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The chemokine receptor D6 limits macrophage uptake of apoptotic PMN, but promotes their immune-silencing and departure during the resolution of inflammation

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Background: D6 is a promiscuous chemokine receptor that binds multiple inflammatory chemokines and clears them from injury sites during the resolution of inflammation.
Materials and methods: Male C57BL/6 mice (6–8 weeks; wild type (WT) or D6-deficient) injected intra-peritoneally (I.P.) with zymosan A. Peritoneal PMN (24 h) were isolated, and incubated O.N. with peritoneal macrophages (66 h). Examination and characterization of CD11b ^{low} macrophages by FACS staining and western blotting was preformed, as well as differences in their responses in the ECIS system.
Results: D6 is expressed on human apoptotic PMN, and that inflammatory peritoneal macrophages from $D6^{-/-}$ mice display higher capacity of apoptotic PMN uptake, accompanied by reductions in their departure of resolving inflammation sites and CD11b downregulation, as well as increased responsiveness to LPS, in comparison to wild type macrophages. Moreover, $D6^{-/-}$ macrophages enhanced lymphoid tissue cellularity upon transfer to WT mice. Notably, although enhanced apoptotic PMN engulfment is mediated by $D6^{-/-}$ macrophages, a defect in the interaction of apoptotic $D6^{-/-}$ PMN with macrophages was also evident.
Conclusions: D6 plays a role in the immune-silencing of macrophages by apoptotic PMN and in the regulation of peritoneal macrophage turnover. These results also suggest that immune-silencing of macrophages blocks further apoptotic PMN engulfment and licenses macrophages to depart resolving inflammation sites, thereby shifting the current paradigm in the resolution of acute inflammation.

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Actin cytoskeleton remodelling in bone resorbing osteoclasts

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Osteoclasts are hematopoietic cells of the monocytic lineage, found on the bone surface, where they participate in the bone remodeling by resorbing its mineralized extracellular matrix. Multinucleated osteoclasts, are formed by fusion of precursors in presence of two cytokines, MCSF and RANKL. They share in common with monocyte-derived cells the ability to phagocytize and to form actin-containing adhesion structures named podosomes when adherent on glass or plastic. Podosomes are formed by a core of F-actin surrounded by a cloud of loose F-actin. They collectively self assemble during osteoclast differentiation. When adherent on bone, their natural substratum, osteoclasts polarize and podosome structures condense to form a so called 'sealing zone' delimiting the resorption area. We will present the dynamic actin remodeling during these different phases of osteoclast activities and will discuss the different role played by podosomes in osteoclast adhesion, bone degradation and migration.

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The PI3-kinases p110β and p110δ regulate osteoclast development, function and actin ring formation

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Background: Osteoclasts are bone-resorbing cells of myeloid origin. Class I PI3-kinases (p110α,β,γ,δ) have crucial roles in regulating a variety of cellular functions, but their role in osteoclast biology is poorly understood. Here we tested the role of p110β and p110δ in human and murine osteoclast development and function using combined genetic and pharmacological approaches.

Materials and methods: Murine bone marrow cells were isolated from wild-type, $p110\beta^{-/-}$ or $p110\delta^{KinaseDead/KinaseDead}$ mice and differentiated into osteoclasts *in vitro* in the presence of murine M-CSF and RANKL. Human monocytes were purified from whole blood of healthy volunteers and differentiated into osteoclasts *in vitro* in the presence of human M-CSF and RANKL. Osteoclast differentiation and function were examined by TRAP-staining and resorption of artificial hydroxyapatite. PI3-kinase inhibitors TGX221 and IC87114 were used as selective inhibitors of p110β and p110δ. For analysis of gene expression, total cellular RNA was extracted, cDNA were synthesized and multiplex PCR was performed with specific probes for the gene of interest. For actin ring formation assays, cells were fixed and stained with Alexa488-Phalloidin.

Results: Pharmacological inhibition of p110β and/or p110δ resulted in impaired osteoclast differentiation, as evidenced by decreased number of TRAP-positive cells and resorption in *in vitro* human and murine osteoclast cultures. Genetic deficiency of p110β or p110δ also led to impaired osteoclast formation, resorption and actin ring formation in *in vitro* murine osteoclast cultures. The effect of the inhibition or deficiency of p110β consistently triggered stronger inhibition of osteoclast development than those of p110δ. The decreased osteoclast formation was not accompanied by down-regulated expression of osteoclastogenic genes.

Conclusions: The PI3-kinases p110β and p110δ are required for osteoclast development and function by regulating differentiation, bone resorbing activity and cytoskeletal organization.

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Signaling through β2 integrins modulates neutrophil apoptosis and the resolution of inflammation

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Background: Timely removal of neutrophils from inflamed tissues is essential for efficient resolution of inflammation. Emerging data indicates a role for the β2 integrin Mac-1 in modulating neutrophil life span. Since Mac-1 also binds the neutrophil granule constituent myeloperoxidase and Mac-1expression is regulated by aspirin-triggered 15-epi-lipoxin A_4, we studied Mac-1 modulation of neutrophil apoptosis and outcome of inflammation.

Methods: Human neutrophil apoptosis was studied in the presence of myeloperoxidase with or without 15-epi-lipoxin A_4. Acute lung injury was produced by intratracheal instillation of carrageenan plus myeloperoxidase or intraperitoneal injection of live *Escherichia coli* in mice, and the animals were treated with 15-epi-lipoxin A_4 at the peak of inflammation.

Results: Myeloperoxidase signalling through Mac-1 rescued neutrophils from constitutive apoptosis via ERK and Akt-mediated phosphorylation of Bad and preservation of Mcl-1 expression and prolonged neutrophil-dependent lung injury in mice. In human neutrophils, 15-epi-LXA_4 attenuated myeloperoxidase-induced upregulation of Mac-1 expression and overcame the powerful anti-apoptosis signal from myeloperoxidase. 15-epi-LXA_4 promoted neutrophil apoptosis by attenuating myeloperoxidase-evoked ERK and Akt signalling, leading to acceleration of Mcl-1 degradation. These led to collapse of mitochondrial transmembrane potential and subsequent activation of caspase-3. 15-epi-LXA_4 also interrupted a myeloperoxidase-mediated autocrine/paracrine amplification circuit for Mac-1 signaling in neutrophils. In mice, treatment with15-epi-LXA_4 accelerated the resolution of established carrageenan plus myeloperoxidase-evoked and live *E. coli*-induced pulmonary inflammation through reducing pulmonary neutrophil accumulation, redirecting neutrophils to caspase-3-mediated apoptosis and enhanced their phagocytosis by macrophages.

Conclusions: These results demonstrate that myeloperoxidase-triggered Mac-1-mediated outside-in signaling suppresses neutrophil apoptosis and prolongs inflammation, whereas aspirin-triggered 15-epi-LXA_4 overrides myeloperoxidase signaling and enhances the resolution of acute lung inflammation by redirecting neutrophils to apoptosis. (Grant support: CIHR MOP-64283 and MOP-97742).

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The role of CARD9 in phagocytes and in experimental arthritis

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Background: CARD9 is a caspase recruitment domain containing intracellular adaptor protein that is highly expressed in myeloid cells. In contrast to its well-identified role in antimicrobial defense, the function of the protein in non-infectious inflammation has not been described yet. Here, we investigated the role of CARD9 in an autoimmune model of arthritis by a genetic approach.

Materials and methods: For the *in vitro* studies, neutrophil granulocytes were isolated, macrophages were cultured from the bone marrows of wild type and CARD9 KO mice. These phagocytes were plated on an immobilized immune complex surface. From the triggered cell responses, the superoxide release (as a *short-term response*), the phosphorylation/degradation of IκBα, the NFκB-activation and the cytokine release (as *long-term responses*) were detected. During our *in vivo* experiments, we used the K/BxN serum transfer arthritis model (in the development of which neutrophils, macrophages and Fc receptors are crucial) in the presence and in the absence of CARD9.

Results: Despite of the fact that both the wild type and the CARD9 knockout neutrophils and macrophages were able to produce the same amount of superoxide when stimulated

through their Fc receptors, the long-term responses of the knockout cells were greatly reduced. The severity of arthritis showed a partial decrease in the CARD9 KO mice compared to the wild type animals.

Conclusions: According to our results, CARD9 was important in the development of autoimmune arthritis and this intermediate inflammation state was probably due to the strong reduction of long-term cell responses of CARD9 knockout neutrophils and macrophages.

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Signal transduction in autoimmune inflammation

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Background: Signal transduction by classical immunoreceptors (B- and T-cell receptors and Fc-receptors) is mediated by phosphorylation of receptor-associated immunoreceptor tyrosine-based activation motif (ITAM) containing adapter molecules by Src-family kinases, followed by SH2 domain-mediated recruitment of the Syk or ZAP70 kinase and downstream signaling by members of the phospholipase Cγ (PLCγ) family. For a long time, this pathway was thought to be specific for classical immunoreceptors. The aim of the present study was to identify additional biological functions mediated by the above immunoreceptor-like signal transduction pathway.

Methods: Mice genetically deficient of various signal transduction molecules were subjected to *in vitro* analysis of various phagocytic cell functions, as well as to the K/BxN serum transfer arthritis, an *in vivo* model of autoimmune inflammatory arthritis.

Results: β_2 integrin-mediated neutrophil activation required Src-family kinases, the ITAM-containing DAP12 and FcRγ adapter proteins, the Syk tyrosine kinase and PLCγ2. The same molecules also participated in β_2 integrin-mediated activation of macrophages. DAP12/FcRγ, Syk and PLCγ2 were also required for osteoclast development and osteoclast-mediated bone resorption. The human genome was found to contain an unexpectedly large number of ITAM-containing molecules. Src-family kinases, Syk and PLCγ2 were all required for the development of the K/BxN serum-transfer arthritis.

Conclusion: Our studies indicate that signal transduction pathways reminiscent of those utilized by classical immunoreceptors also mediate non-immunoreceptor signaling processes in diverse biological functions. In addition, this same signal transduction pathway also participates in pathological processes, such as the development of autoantibody-induced autoimmune arthritis. Components of this pathway may prove to be suitable targets of the pharmacological therapy of autoantibody-mediated diseases such as certain subsets of rheumatoid arthritis.

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A dithiol – disulfide switch in the cytosolic part of Nox2 controls NADPH oxidase assembly

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Background: The cytosolic segment of human Nox2 contains a Cys-Gly-Cys triad (^{369}CGC^{371}), conserved in Nox2 of all species but absent in Nox1, 3, 4, and 5. A C369R mutation results in an X91$^+$ form of chronic granulomatous disease. The purpose of this study is to establish the effect of the redox state of the cysteines on NADPH oxidase assembly.

Materials and methods: We developed an ELISA to identify the binding site(s) for p67^{phox} in the cytosolic tail of Nox2, based on the binding of 6His-tagged p67^{phox} to biotinylated peptides, corresponding to Nox2 residues 288–570, attached to streptavidin-coated wells.

Results: (i) p67^{phox} bound to Nox2 peptides 357–371 and 369–383, sharing residues ^{369}CGC^{371}; (ii) 'Mutating' C369 or C371 to R, in the two peptides, eliminated binding of p67^{phox}; (iii) Exposing the peptides to the reducing agent dithiothreitol (DTT) or to reduced glutathione (GSH) abolished binding of p67^{phox}, demonstrating the involvement of a disulfide bond between C369 and C371 in binding; (iv) Reestablishing the disulfide bond by treatment of the DTT-reduced peptides with diamide restored binding of p67^{phox}; (v) Alkylation of DTT-reduced cysteines with *N*-ethylmaleimide or iodoacetamide perpetuated the lack of binding; (vi) Introducing an intramolecular disulfide bond between C369 and C371 during peptide synthesis, resulted in a marked increase in binding of p67^{phox}, which was abolished by DTT or GSH and could be restored by diamide; (vii) The effect of diamide was mimicked by phenylarsine oxide and by the fungal toxin, gliotoxin.

Conclusions: The ^{369}CGC^{371} triad, located between the FAD- and NADPH-binding regions of Nox2, exists in a dynamic state, switching from dithiol to disulfide forms. The redox state governs the binding and dissociation of p67^{phox}.

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Role of the putative second transmembrane region ^{45}LLGSALALARAPAACLNFNCMLILL^{69} of Nox2 in the structural stability and electron transfer in the phagocytic NADPH oxidase

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Background: Flavocytochrome b (Cytb) of phagocytes is an integral membrane, heterodimeric protein comprised of two subunits, p22^{phox} and gp91^{phox}. The latter subunit, also known as Nox2, has a cytosolic C-terminal 'dehydrogenase domain' containing FAD/NADPH binding sites. The N-terminal half of Nox2 contains six predicted transmembrane α-helices coordinating two membrane hemes. We studied the role of the second transmembrane α-helix, which contains a 'hot-spot' for mutations of rare X$^+$ and X$^-$ chronic granulomatous disease.

Materials and methods: By site-directed mutagenesis and stable transfection in the X-CGD PLB-985 cell line, we examined the functional and structural impact of seven missense mutations on five residues (P56L, C59F, A53D, R54G/M/S, A57E) of Nox2, in intact cells, purified membranes and immuno-purified cytb.

Results and conclusions: P56L and C59F mutations drastically affect the level of Nox2 expression indicating that these residues are important for the structural stability of Nox2. A53D, R54G/M/S mutations do not affect Nox2 expression, spectral properties of oxidized and reduced cytochrome b_{558}, oxidase complex assembly, FAD binding, nor INT reductase (diaphorase) activity, but inhibit superoxide production. This suggests that A53 and R54 are essential to control the electron transfer occurring from FAD. Surprisingly A57E mutation partially inhibits FAD binding, diaphorase activity, oxidase complex assembly and affects the affinity constant for p67^{phox} of the

immuno-purified A57E cytochrome b_{558}. This suggests that the A57E mutation directly or indirectly disturbs the integrity of the 'deshydrogenase domain' of Nox2 and emphasizes that oxidase assembly and FAD binding are related events controlling the diaphorase activity. Finally by comparing iodonitrotetrazolium reductase activity of immuno-purified mutated and wild-type cytb under aerobiosis vs. anaerobiosis we showed that this activity reflects the electron transfer from NADPH to FAD only in the absence of superoxide production.

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Phagocyte signaling by 14-3-3 proteins regulates NADPH oxidase 2

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Background: We present a novel signaling mechanism by which reactive oxygen species (ROS) generation by NADPH oxidase 2 (Nox2) in human neutrophils is regulated by 14-3-3 proteins.
Materials and methods: Human neutrophils were used for co-immunoprecipitation studies. Human embryonic kidney cells (HEK 293 cells) were transfected with Nox2, $p47^{phox}$, $p67^{phox}$, constitutively active Rac1G12V, and either 14-3-3 WT, 14-3-3 dominant negative mutants, or 14-3-3 isoform-specific siRNA. A luminol-based chemiluminescent assay was used to measure ROS generated by neutrophils or transfected cells. Phorbol myristate acetate (PMA) or bacterial peptide, fMetLeuPhe (fMLF), was used to stimulate the cells.
Results: The mutation, K50E, of the 14-3-3 gamma dimer has a dominant negative (DN) effect that inhibits binding of the WT dimer to its client phosphoproteins due to the formation of a mutant-WT dimer. Co-expression of 14-3-3 gamma DN with Nox2 and its regulatory subunits in HEK 293 cells increased ROS production by a remarkable 4–5- fold compared to 14-3-3 WT under PMA-stimulated conditions. Knockdown of endogenous 14-3-3 gamma in HEK 293 cells using 14-3-3 isoform-specific siRNA increased ROS production by 2–3 fold compared to a control siRNA. In human neutrophils, endogenous 14-3-3 gamma co-immunoprecipitated with endogenous phosphorylated $p47^{phox}$ under PMA or fMLF stimulated conditions.
Conclusion: These results suggest that 14-3-3 gamma functions as a negative regulator of ROS production by Nox2. The 14-3-3 gamma isoform appears to interact directly with phosphorylated $p47^{phox}$ and may act as a dampener of ROS generation by Nox2 by sequestering phosphorylated $p47^{phox}$ in the cytosol, thereby limiting translocation of $p47^{phox}$ to the membrane.

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Legionella pneumophila modulates the expression of the inflammasome members to establish infection in human monocytes

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Background: Mice and their derived macrophages are resistant to the intracellular pathogen *Legionella pneumophila*. In murine cells, the activation of the inflammasome, which mediates a unique inflammatory response, is sufficient and essential to restrict infection. In contrast, human monocytes and macrophages allow *Legionella* growth resulting in pneumonia. For unknown reasons, the inflammasome activation in primary human monocytes is suppressed during *Legionella* infection. Therefore humans are susceptible to infection while mice are resistant.
Material and methods: The mRNA level of essential inflammasome members was evaluated by RT-PCR and expression of corresponding proteins were examined by Western blot. Functional assays and permissiveness to infection were evaluated following over expression and depletion of specific inflammasome members by small interfering RNA (siRNA).
Results: The adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc) is down regulated in primary human monocytes but not in murine cells. Restoration of ASC levels in human monocytes during *Legionella* infection permitted the inflammasome to respond efficiently to *Legionella* and to restrict infection. On the other hand, further depletion of endogenous ASC in human monocytes allowed extensive growth of the pathogen. Moreover, the levels of ASC expression inversely correlated with the activation of NF-kB, a host survival pathway required to give time for *Legionella* to establish infection and replicate intracellularly.
Conclusions: We demonstrate for the first time how a pathogen down regulates an essential adaptor molecule to modulate the activation of the inflammasome and the NF-kB pathway to its advantage to establish residency within human phagocytes.

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Role of ion channels in phagocytosis and bacterial killing

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Phagocytosis is a fundamental cellular activity that enables cells of the immune system to engulf and eliminate microbes or other foreign particles by a coordinated sequence of oxidative and lytic processes. During phagocytosis, superoxide radicals are produced within the phagosome as electrons are transported across the phagosomal membrane by the NADPH oxidase (NOX), a process that releases large quantities of acid into the cytosol. Ion channels are required to maintain a permissive membrane potential that enables the sustained transfer of electrons by the voltage-dependent NOX, and efficient acid extrusion mechanisms must protect phagocytic cells from excessive acidification. Studies from animal models indicate that the voltage-gated proton channel Hv1 (also known as voltage-sensing domain only protein or VSOP) promotes superoxide production by preventing the depolarization and mitigating the acidification during the respiratory burst. Potassium channels do not contribute significantly to charge compensation and their role in the maintenance of the ionic homeostasis of the phagosome must be re-evaluated. By compensating the charge generated by the oxidase at the plasma membrane, proton channels not only sustain the oxidase activity but also favour the entry of calcium ions that control the adhesion and motility of neutrophils (El Chemaly J. Exp. Med. 2010). Non-selective cationic channels have the opposite effect as the entry of cations depolarizes cells, thereby inhibiting oxidase activity and reducing the driving force for calcium entry. On the other hand, depolarization of the plasma membrane boosts phagocytosis by increasing the synthesis of phosphatidylinositol-4,5-bisphosphate and the depolymerisation of actin, a mechanism that accounts for the impaired phagocytosis of

macrophages lacking the cation channel TRPV2 (Link, Nat. Immunol. 2010). Finally, the ER calcium sensor STIM1 and the store-operated calcium channel Orai1 are both recruited to phagocytic cups and ectopic STIM1 expression improves phagocytosis, suggesting that store-operated calcium channels participate in the regulation of phagosome formation and maturation. Ion channels therefore control phagocytosis in multiple ways via their impacts on cell signalling and on the ionic homeostasis, membrane voltage, and lipid composition of phagosome.

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Role of the small GTP-binding protein RhoG in neutrophil biology

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Background: Reactive Oxygen Species (ROS) are an important weapon used by neutrophils and other 'professional' phagocytes to protect host tissues from infection. Conversely, deregulated ROS secretion by neutrophils has been implicated in autoimmune disease. Synthesis of ROS is driven by a multisubunit protein complex known as the NADPH oxidase, and is regulated by the activity of Rho family GTP-binding proteins (G proteins) and the cooperative engagement of their associated guanine nucleotide exchange factors (GEFs). The Rac-related small G protein RhoG has been shown to regulate fMLF-induced ROS production in primary murine neutrophils, although the mechanisms through which RhoG exerts its role have not been characterized. This study aims to characterize the role of RhoG in signalling to the NADPH oxidase and gain novel insights into the complex web of G protein-GEF interactions which regulate signalling in neutrophils.
Methods: We have used biochemical and genetic approaches in order to characterize the role of RhoG in primary murine neutrophils.
Results: RhoG is activated downstream of the formyl peptide and Fc receptors and is required for NADPH oxidase activity. In addition RhoG regulates integrin-mediated ROS secretion and spreading but not ROS secretion into phagosomes. RhoG-deficient neutrophils display substantially reduced Rac activity, thus providing a mechanistic basis for the functional defects in these cells.
Conclusions: RhoG regulates a number of neutrophil processes, including the NADPH oxidase via Rac-dependent mechanisms. Measures of RhoG activity in neutrophils from GEF-deficient mice are beginning to reveal the extent to which RhoGEFs regulate Rac through a RhoG-mediated signalling axis, rather than the direct Rac-GEF activity suggested by previous studies.

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Heme induces macrophage death via tumor necrosis factor and reactive oxygen species production

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Background: Diseases that cause hemolysis or myonecrosis lead to the leakage of large amounts of heme proteins. Free heme has proinflammatory and cytotoxic effects. The cytotoxicity of heme has been attributed to its ability to intercalate into cell membranes and cause oxidative stress through the Fenton chemistry. We recently described that heme induces TLR4-dependent production of TNF.
Materials and methods: Heme was purchased from Frontier Scientific, NAC, apocynin and Necrostatin-1 were obtained from Sigma-Aldrich. Recombinant mouse TNF was from Peprotech. Peritoneal macrophages were obtained by instillation of thioglycollate 3%. Cell death was analyzed by LDH and MTT.
Results: Heme caused macrophage death after 6 h, albeit in serum-free condition. Heme-induced cell death was increased in macrophages from heme oxygenase-1 deficient (*Hmox−/−*) mice, while it was abolished in *Tlr4−/−*, Myd88−/− and in *TnfrI−/−* macrophages. Addition of TNF to *Tlr4−/−* or to *Myd88−/−* macrophages restored heme-induced cell death. Antioxidants also abrogated the macrophage cell death induced by heme, however this effect was not reversed by the addition of exogenous TNF. The use of a selective RIP-1 inhibitor, necrostatin-1, or cells deficient on *Rip1−/−* or *Rip3−/−* revealed a critical role for Rip proteins in heme-induced cell death. Treatment with Nec-1 did not interfere with the generation of ROS by heme.
Conclusions: These results reveal that heme-induced macrophage cell death is mediated by two independent pathways triggered by TNF and ROS.

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The microRNA repertoire of human monocytes and neutrophils: induction by pro and antinflammatory signals

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Background: In this study, we investigated the involvement of miRNAs in the regulation of human polymorphonuclear neutrophils (PMN) and monocytes responses to classical pro and antinflammatory stimuli, such as LPS and IL-10.
Methods: TaqMan-based Array and RT-qPCR were employed to study miRNAs expression. Predicted miRNA targets were validated in 3′UTR-luciferase reporter cotransfection experiments and in primary monocytes in which the selected miRNA were either overexpressed or silenced.
Results: RT-qPCR-based high throughput screening allowed us to identify miR-146, miR-155, miR-132, miR-187, miR-125a/miR-99b/let-7e cluster, and miR-9/9* as LPS-responsive miRNAs. In particular, we discovered miR-9 as the only miRNA upregulated both in PMN and monocytes by LPS in a MyD88- and NF-κB-dependent manner. By blocking endogenous IL-10 action we found that the expression of LPS-induced miR-9, miR-155, miR-132 and miR-146a was strongly increased and, most importantly, that miR-187 was the only miRNA induced by LPS in an IL-10-dependent manner.

Luciferase reporter assays together with overexpression and/or silencing of miR-9 or miR-187 provided experimental evidence validating the *in silico* predicted NFKB1/p50 and NFKBIZ as miR-9 and miR-187 targets, respectively.
Conclusion: In the present study, we provide the first evidence linking miR-9 and miR-187 to the innate immune response and we identified their relative target genes. Overall, these data suggest that TLR4-activated NF-κB rapidly increases the expression of miR-9 that operates a feed-back control of the NF-κB-dependent responses by fine tuning the expression of a key member of the NF-κB family. Additionally, we discover that IL-10 strongly potentiate LPS-induced miR187

expression, that, in turn, feedback regulates the levels of IkBzeta, a transcription factor essential for the induction of IL-6.

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The involvement of neutrophils in cross-talks with immune cells

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Neutrophils have been for long considered as innate immune cells able to exert only antiinfectious and pro-inflammatory functions, due to their ability to phagocytose and produce very powerful antimicrobial peptides, proteolytic enzymes and reactive oxygen intermediates. Nevertheless, it is nowadays accepted that the role of neutrophils goes far beyond phagocytosis and pathogen killing. A fascinating aspect that has gradually come to light in the last two decades is the ability of neutrophils to newly express a number of genes, whose products, including a variety of cytokines and chemokines, lie at the core of inflammatory and immune responses. Consequently, researches stimulated by the latter observations have provided unequivocal evidence that neutrophils, by producing and releasing cytokines, may significantly contribute to the regulation of many different processes and, in turn, act as key regulators of cross-talks among endothelial, stromal and parenchymal cells. More recent demonstrations that neutrophils also undertake bidirectional cross-talks with immune cell types, including dendritic cells, NK cells, iNKT cells, as well as unpolarized/polarized T cells, further emphasizes their previously unsuspected capacity to more consistently contribute as active orchestrators of innate and adaptive immunity. Given their peculiar role in inflammation, clearance of pathogens/viral-infected cells, and cancer immunosurveillance, the current knowledge about the mechanisms whereby of neutrophils, dendritic cells and NK cells interact and regulate the activities of one another, as well as their potential implications involved in the pathogenesis of chronic inflammatory pathologies, will be discussed.

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The novel Rac GTPase activating protein ARHGAP25 regulates phagocytosis in neutrophils

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Background: Members of the Rho family small GTPases play essential role in the signal transduction of innate immunity. GTPase activating proteins (GAPs) decrease the amount of the GTP-bound, active form of small GTPases and contribute to the termination of biological signals. Our previous data show that ARHGAP25 is specifically expressed in haematopoietic cells and acts as a RacGAP *in vitro*. We found increased F-actin level and serum-opsonized zymosan (OPZ) uptake in ARHGAP25-silenced PLB cells. The aim of our study was to specify the role of ARHGAP25 in neutrophils.
Materials and methods: *In vivo* GAP activity was measured in ARHGAP25-overexpressing COS7 cells. ARHGAP25-transfected COS*phox*FcγR cell lines were used for functional studies. We prepared the loss-of-function mutant ARHGAP25R192A to investigate the role of GAP activity in phagocytosis. We investigated the *in vitro* phosphorylation using ^{32}P-ATP and lipid-binding ability of recombinant ARHGAP25 in PIP Strip assay.
Results: We found that ARHGAP25 acts as a RacGAP *in vivo* as well. In COS*phox*FcγR cells, overexpression of the protein blocked phagocytosis. Mutation of the critical arginine in the GAP domain abolished this inhibitory effect. ARHGAP25 showed non-specific binding to phosphatidyl-inositols and the isolated Coiled Coil domain bound specifically to $PI(4,5)P_2$. Addition of neutrophil cytosol caused phosphorylation of recombinant ARHGAP25 which effect could be partially abolished by PKC inhibitors.
Conclusion: We suggest that ARHGAP25 RacGAP regulates phagocytosis of neutrophils by controlling Rac-dependent F-actin reorganisation. Phosphorylation and binding to phosphatidyl-inositols are the possible regulating mechanisms of ARHGAP25.

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Stomal interaction molecule 1 -mediated recruitment of endoplasmic recticulum membranes to nascent phagosomes

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Background: Endoplasmic recticulum (ER) recruitment as well as Ca^{2+} signalling have both been implicated in phagocytosis, however their respective roles in this important process are not fully understood. The present study sought to investigate whether a link between ER remodelling and Ca^{2+} signalling events during phagocytosis could occur via the ER-resident store-operated Ca^{2+} entry (SOCE) sensor stomal interaction molecule 1 (STIM1).
Materials and methods: STIM1 knockout mouse embryonic fibroblasts (MEFs) were generated by insertional mutagenesis, and were rendered phagocytic by ectopic expression of myc-tagged Fc-gamma-RIIA receptors. Confocal and spinning-disk fluorescence as well as electron microscopy were used to visualize cells.
Results: STIM1 knockout MEFs were found to have a 10 fold decreased SOCE as compared to wild type. Transfecting STIM1 knockout MEFs with STIM1-YFP rescued SOCE by approximately fivefold. STIM1 overexpression or rescue enhanced the phagocytic index in wild type and STIM1 knockout cells respectively. Electron microscopic analysis revealed that STIM1 is recruited to the base of phagocytic cups and to early phagosomes, forming tight phagosomal-ER junctions. Additionally, when STIM1-YFP dynamics were followed in living neutrophil-like HL60 cells, STIM1 was observed to localize to cups and early phagosomes, disappearing from the vicinity of phagosomes approximately 15 min after enclosure. Ectopic expression of EGFP-tagged partner SOCE channels Orai1, 2 and 3, in both phagocytic MEFs as well as HL60, all localized to cups and early phagosomes, whereas Orai2 and 3 but not Orai1 remained on phagosomes at later time points.
Conclusions: These data suggest that formation of STIM1-mediated ER-phagosomal junctions as well as subcellular targeting of Orai family channels to phagosomes may provide a mechanism that drives localized Ca^{2+} elevations during phagocytosis.

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Role of the lysosomal calcium sensor synaptotagmin VII in membrane traffic and phagocytosis

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Conventional lysosomes in many cell types have the ability to respond to intracellular free calcium elevations by fusing with the plasma membrane. We identified in lysosomes a member of the synaptotagmin family of calcium sensors, Syt VII, which regulates lysosomal exocytosis and also the delivery of lysosomal membrane to nascent phagosomes in macrophages. Using live imaging we found that Syt VII and the lysosomal tetraspannin CD63 are rapidly delivered to the plasma membrane at the onset of phagocytosis, while the lysosomal glycoprotein Lamp1 remains associated with tubular lysosomes that extend towards the particle being phagocytosed. Lamp1 is eventually delivered to recently-formed phagosomes, but this process lags behind the rapid delivery of Syt VII and CD63. We found that Syt VII and CD63 form a molecular complex that depends on palmitoylation, and that is responsible for targeting Syt VII to lysosomes. Syt VII is retained in the Golgi complex when its palmitoylation sites are mutated, and when expression of CD63 is silenced by RNAi. These results clarify the mechanism by which Syt VII traffics to lysosomes, and provide insight into how Syt VII and CD63 are simultaneously mobilized to nascent phagosomes.

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Intracellular pathogens interfere with the IFN-γ-signaling in neutrophil granulocytes

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Background: The intracellular pathogens *Anaplasma phagocytophilum* (*A.p.*) and *Leishmania major* (*L.m.*) survive inside neutrophils. Since transcriptome analysis revealed diminished expression of IFN-γ-regulated genes in infected neutrophils we hypothesized that the pathogens interfere with the IFN-γ-signaling pathway in neutrophils.
Materials and methods: Human primary neutrophils were infected with *A.p.* and *L.m.*, exposed to IFN-γ, and the functionality of the IFN-γ signaling pathway was analysed *in vitro*.
Results: As read out for intact IFN-γ signaling, the secretion of the IFN-γ-inducible chemokines IP-10/CXCL10 and MIG/CXCL9 was assessed. Infection with both *A.p.* and *L.m.* markedly inhibited the release of these chemokines by neutrophils. Molecular analyses revealed that infection with *A.p.* and *L.m.* resulted in a decreased expression of the IFN-γ receptor α-chain CD119 and diminished IFN-γ-induced phosphorylation of STAT1. Moreover, and enhanced the expression of SOCS1 and SOCS3 in neutrophils both at mRNA and protein level.
Conclusions: Since IFN-γ activates various antimicrobial effector mechanisms of neutrophils, the impaired IFN-γ signaling in infected cells likely contributes to the survival of *Anaplasma phagocytophilum* and *Leishmania major* inside PMN and in turn to disease development.

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The effect of CD40/CD40Ligand interactions on human bone marrow granulopoiesis

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Background: CD40 induces diverse biologic responses related to cell survival and growth upon interaction with its ligand (CD40L). Because reduced expression of CD40L has been associated with neutropenia, we investigated the role of CD40/CD40L interactions on granulopoiesis.
Materials and methods: CD40 expression was assessed on immunomagnetically sorted $CD34^+$, $CD34^-/CD33^+$ and $CD34^-/CD33^-/CD15^+$ normal bone marrow (BM) cells, representing sequential stages of the granulocytic development,. We evaluated the proportion of apoptotic cells in the above cell populations following CD40 activation and investigated the effect of CD40L on the production of granulopoiesis-promoting cytokines by long-term BM culture stromal layers.
Results: CD40 was minimally expressed on the $CD34^+$, $CD34^-/CD33^+$, $CD34^-/CD33^-/CD15^+$ but was substantially induced in the presence of TNFα. Cross-linking of CD40 in the above cell populations resulted in induction of apoptosis that was further enhanced in the presence of Fas ligand. CD40 activation in the above cell populations resulted in Fas up-regulation, providing a possible mechanism of the CD40-mediated apoptotic effect. Addition of CD40L in clonogenic assays resulted in a significant decrease in the colony-forming capacity of BM mononuclear cells from patients with chronic idiopathic neutropenia presumably expressing high levels of CD40 in the progenitor cells. This effect was reversed upon CD40 blockade, indicating the pathophysiologic significance of the CD40/CD40L interaction. CD40 was constitutively expressed on normal long-term BM cultures stromal cells and upon activation resulted in increase in granulocyte-colony stimulation factor and granulocyte/macrophage-colony stimulating factor production.
Conclusions: CD40/CD40L interactions indirectly promote granulopoiesis under steady state conditions by inducing the stromal release of granulopoiesis-supporting cytokines whereas under inflammatory conditions they directly affect the granulocytic progenitor/precursor cell survival by accelerating Fas-mediated apoptosis.

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Modulation of pro-inflammatory response of monocytes by glucocorticoids

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Background: We previously demonstrated that the treatment of monocytes with GC does not suppress monocytes functions, but rather induces specific differentiation of cells with

anti-inflammatory phenotype. Nevertheless the mechanism of GC action on monocytes during inflammation is currently not well defined. The aim of our studies was to investigate the effects of GC on pro-inflammatory monocytes.
Materials and methods: GC-, LPS- and GC-LPS-induced gene expression patterns in monocytes were analysed by microarray technology. The results were independently confirmed by real-time PCR. Protein expression and activation was analysed by Western Blot and Flow Cytometry. Monocyte functions pivotal for innate immunity – migration, chemotaxis, phagocytosis, killing, oxidative burst – were assessed.
Results: As expected, treatment of LPS-stimulated monocytes with GC resulted in inhibited expression of many of pro-inflammatory factors. Nevertheless, many of them have not been described to be regulated by GC in monocytes so far. Surprisingly, we have found that GC-LPS treatment of monocytes led also to synergistical up-regulation of many genes, which are not induced by GC or LPS alone. Moreover, we observed additive effects of GC and LPS on expression of many anti-inflammatory genes which was much more pronounced compared to monocytes stimulated with GC alone. Analysis of the specific function of monocytes have shown that GC-LPS treatment inhibited specifically monocyte adherence, but enhanced spontaneous migration, chemotaxis, phagocytosis and killing of pathogens, and engulfment of apoptotic cells as well as the ability to produce anti-inflammatory lipid mediators.
Conclusions: Our results demonstrate that GC do not simply suppress LPS-mediated activation of monocytes, but rather induce their re-programming toward a specific anti-inflammatory phenotype involved in resolution of inflammation.

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Human macrophage polarization and HIV infection. Multiple restriction pathways

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Background: Human mononuclear phagocytes are crucial targets for HIV infection and pathogenesis. Unlike CD4+ T cells, infection of mature macrophages is not cytopathic resulting in long-term accumulation of virions in intracytoplasmic vacuoles (a feature not observed in T cells) for which these cells have been defined as 'Trojan horses' of HIV. The role of macrophage polarization is poorly understood.
Materials and methods: Human monocyte-derived macrophages (MDM) were obtained by HIV-negative, healthy donors and infected *in vitro* with CCR5-dependent (R5) HIV-1 strains following 18 h of polarization towards M1 (TNF-a + IFN-g) or M2a (IL-4) vs. control. Virus replication has been monitored by published methods.
Results: Both classical (M1) or alternatively activated (M2a) MDM show a reduced capacity to support productive R5 HIV-1 infection. M1 cells show a significant downregulation of CD4 a > 90% decrease in HIV-1 DNA levels 48 h post-infection. In contrast, M2a polarization had no effect on either HIV-1 DNA or protein expression levels, indicating that the inhibitory effect occurred at late/post-integration levels in the viral life cycle. Most phenotypic and functional changes are fully reversible 7 days after removal of the polarizing stimulus and a reciprocal downregulation of M1-related chemokines and cytokines was observed in M2a-MDM and vice versa. See also: E. Cassol et al., J Immunol. 182: 6237, 2009.
Conclusions: M1/M2a polarization may represent a mechanism that allows macrophages to cycle between latent and productive HIV-1 infection.

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Phagocytosis and activation are impaired in HIV-1 infected macrophages

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Background: Phagocytosis in macrophages is receptor-mediated and relies on actin polymerization coordinated with the focal delivery of intracellular membranes that is necessary for optimal phagocytosis of large particles. The closed phagosomes then undergo maturation and fusion with lysosomes that allow the degradation of the ingested material. Depending on the receptor stimulated, phagosomes maturation is connected to macrophage activation, reactive oxygen species and cytokines secretion. HIV-1 targets macrophages and
Materials and methods: We used primary human macrophages infected or not with HIV-1.
Results: We have recently shown that phagocytosis by various receptors was inhibited in primary human macrophages infected with wild type HIV-1 but not with a nef-deleted virus. We observed no major perturbation of F-actin accumulation but adaptor protein 1 (AP1)-positive endosome recruitment was inhibited in HIV-1-infected cells. Expression of Nef was sufficient to inhibit phagocytosis, and association of Nef with AP complexes were important for this inhibition. Finally, an alteration of the recruitment of VAMP3- and TNFa-positive recycling endosomes regulated by AP1, but not of VAMP7-positive late endosomes, was observed in phagocytic cups of HIV-1-infected macrophages. Therefore, HIV-1 impairs optimal phagosome formation through Nef-dependent perturbation of the endosomal remodeling relying on AP1. We are now analyzing further whether and how the later steps of phagocytosis, i.e. phagosome maturation and macrophage activation, are impaired in HIV-1-infected macrophages using both model particles and intracellular bacteria known to be implicated in opportunistic diseases in HIV-1 infected patients.
Conclusion: Our data reveal how macrophages functions are impaired upon HIV-1 infection and should help us to better understand how opportunistic diseases develop in patients.

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Chronic granulomatous disease caused by a partly exonised retroposed *TMF1* gene copy inserted into the *CYBB* gene

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Background: The NADPH-oxidase complex catalyzes the formation of superoxide, which enables the destruction of pathogens engulfed in phagocytes. An X-linked gene (*CYBB*) and four autosomal genes encode the proteins of this complex. Disabling mutations in *CYBB* lead to gp91-*phox*-deficient X-linked CGD.
Materials and methods: We studied an immunocompromised male individual who suffered from recurrent infections. None of the patient's relatives studied had a health record suggesting an immunodeficiency. The patient was diagnosed with X-CGD.

Results: In the first intron of *CYBB* we detected a novel insertion, which originated either in the maternal germline or during the fetal development of the patient. The insertion arose via retroposition of a partially processed transcript of the *TMF1* gene located on chromosome 3. In the patient we studied, the inserted retrocopy is partly exonised into a defective *CYBB* splice variant carrying a premature stop codon.
Conclusions: We describe the first retroposed gene copy leading to a human disease, which illustrates that although gene retroposition has contributed numerous beneficial novelties during mammalian evolution, it may also produce genetic alterations with immediate and adverse consequences. This is also the first example of a polymorphic retrocopy in man.

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Activation status of neutrophils in the joints of patients with rheumatic disease

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Background: The recruitment of leukocytes into inflammatory foci is a cardinal event in inflammatory responses and inflammatory arthritis (IA) is a group of chronic diseases characterized by invasion of immune cells to the synovial cavity. The majority of cells in the synovial fluid of IA patients are neutrophils. Proinflammatory cytokines and chemokines in the synovial microenvironment are believed to orchestrate the ingress of leukocytes along a chemotactic gradient. Serum amyloid A (SAA), an acute-phase reactant is thought to participate in inflammatory reactions as a neutrophil chemoattractant and inducer of proinflammatory cytokine production. We recently showed that circulating SAA lacked the proinflammatory effects described for the frequently used recombinant form of SAA. Since SAA can also be present in synovial fluids of patients with IA, we explored whether synovial SAA possessed neutrophil activating capacity.
Material and methods: Peripheral blood and synovial fluid was obtained during joint aspiration from patients with IA. SAA was analyzed by a hSAA ELISA. The expression of activation markers on the cell surface was determined by FACS. Cytokines were determined by a multiparameter Luminex bead assay.
Results: SAA was present in synovial fluid of IA patients, albeit at lower levels than in circulation. Neutrophils from synovial fluids surprisingly remained in a resting state despite transmigration from blood to the synovial cavity.
Conclusion: Neutrophils from synovial fluid of IA patients are relatively resting despite having transmigrated from the blood to the synovial cavity in the presence of SAA. These findings imply that endogenous SAA, even outside of circulation, lacks proinflammatory activity.

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Macrophage regulation in atherogenesis

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Macrophages are key determinants in atherosclerosis development. They are not only one of the main cellular constituents of the lesions as foam cells, but also a major source of inflammatory mediators. Thereby they can determine the inflammatory balance during atherogenesis (i.e. atherosclerosis progression or induction of plaque stability), have great impact on activation, migration and survival of other cells in the plaque and thereby ultimately affect clinical outcome of the plaque. The phenotype of macrophages in atherosclerotic lesions is very heterogeneous and can vary dramatically, from a large quiescent lipid-laden foam cell to a small inflammatory active cell. In our research we try to understand the molecular regulation of macrophage function and phenotypes during atherosclerotic plaque development. We focus on inflammatory pathways such as the NF-kB pathway and analyze the role of cytokines, such as IL-10 and type I interferons in atherogenesis. Hereby, we try to identify approaches to modulate macrophages to dampen plaque growth or induce plaque stability.

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The role of CARD9 in host defense against fungal infection: lessons from a CARD9-deficient patient

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Background: CARD 9 is an adaptor molecule containing a N-terminal caspase-recruitment domain (CARD) and a C-terminal coiled-coil domain. Together with Bcl10 and MALT1 CARD9 forms a protein complex in the cytosol of macrophages, neutrophils and myeloid DCs. Studies with CARD9-deficient mice showed that CARD9 controls Dectin-1 mediated Syk-dependent myeloid cell activation, cytokine production and innate anti-fungal immunity. Moreover, CARD9 appears required for the induction of T helper cells producing IL-17 during fungal infection.
Methods: We investigated a patient diagnosed with a rare invasive form of *Candida dubliniensis* radiculo-menigo-encephalitis. We performed sequence analysis of CARD9 gene and western blotting for protein expression. Furthermore, we analyzed the differentiation of T lymphocytes towards Th17 cells, a cytokine response of patients PBMC analyzed in response to various stimuli.
Results: We discovered that the patient carried a compound heterozygous mutation in CARD9 that results in the complete loss of protein expression. Our studies show that CARD9 is indispensable for the production of monocyte cytokines, in particular in response to Candida, and a diminished differentiation of CD4 + Th lymphocytes towards IL-17 producing cells. In addition, neutrophils from this CARD9-deficient patient have a defect in Candida killing.
Conclusions: These findings indicate a selective requirement for CARD9 in the host defense against invasive Candida infection in humans, associated with distinct defects in phagocyte and adaptive immunity.

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CD47-SIRPα interactions form a barrier for antibody-mediated tumor cell destruction by phagocytes

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Background: Monoclonal therapeutic antibodies (mAb) act by binding to tumor cells turning them into targets for killing by immune cells. We have discovered that antibody-mediated killing of tumor cells is limited by an intrinsic mechanism involving interactions between CD47, expressed on tumor cells, and the inhibitory receptor SIRPα present on macrophages and granulocytes. The aim of our study is to prove that interference with CD47-SIRPα interactions promote antibody-dependent killing of tumor cells by phagocytes.
Materials and methods: ADCC was measured using an *in vitro* ^{51}Cr-release assay employing human granucytes as effector cells and Trastuzumab-opsonized Her2/Neu-positive breast cancer cell line SKBR3. The role of the CD47-SIRPα interaction was evaluated by blocking CD47 on tumor cells or blocking SIRPα on effectors. We also evaluated the contribution of CD47-SIRPα interactions in a melanoma model using SIRPα-mutant mice.
Results: Interference with CD47-SIRPα interactions employing antagonistic antibodies enhanced the destruction of tumor cells by ADCC *in vitro*. Moreover, SIRPα-mutant mice that lack the cytoplasmic tail, and hence its signaling capacity displayed an increased capacity for antibody-mediated elimination of tumor cells *in vivo*.
Conclusions: These findings indicate that CD47-SIRPα interactions are part of a homeostatic mechanism that limits antibody-mediated killing of tumor cells. Importantly, the blocking of this interaction may enhance the clinical effects of cancer therapeutic antibodies.

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Neutrophils in head and neck cancer: identification of putative immature neutrophilic myeloid-derived suppressor cells in the peripheral blood and pathophysiological relevance of intratumoral granulocytes

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Background: The progression of epithelial cancer is associated with an intense immunological interaction between the tumor cells and immune cells of the host. In tumor-bearing mice, immunosuppressive granulocytic and monocytic myeloid-derived suppressor cells (MDSC) have been identified.
Materials and methods: In this study we investigated PMN-related changes in the peripheral blood of cancer patients (n = 103) and retrospectively analyzed the correlation of intratumoral PMN counts with clinical parameters (n = 99). Cell biological functions of peripheral blood PMN were directly analyzed *ex vivo*. The modulation of PMN by tumor cells was investigated in an *in vitro* co-culture system of blood PMN and head and neck (HNC) cancer cell lines.
Results: In the peripheral blood, we identified and characterized a subset of neutrophils with altered sedimentation properties in density gradients. These neutrophils suppressed proliferation and IFN-γ production of polyclonally stimulated T cells and thus qualify as human MDSC. Immunophenotyping revealed the accumulation of immature PMN in the MDSC fraction. Neutrophilic MDSC showed prolonged survival and impaired effector functions when compared with conventional mature PMN of regular density. Chemotaxis towards tumor-conditioned medium was reduced and MDSC lacked expression of chemokine receptors CXCR1 and CXCR2. *In vitro*, HNC cells enhanced survival of PMN and promoted inflammatory functions such as release of lactoferrin, MMP-9 and CCL4. Analysis of patient cohorts revealed that higher intratumoral PMN counts were associated with advanced disease and poor survival.
Conclusions: Our results suggest distinct and pathophysiological relevant alterations of PMN in the peripheral blood and the malignant tissue of cancer patients.

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Antibacterial microvesicles from neutrophilic granulocytes

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Background: Microvesicles (MV) are involved in important processes, such as coagulation, signal-, protein and siRNA transfer, or antigen presentation. We investigated formation, components and biological effects of neutrophilic granulocites (PMN) derived vesicles.
Materials and methods: PMN were prepared from blood of healthy volunteers. After incubating PMN with activator, cell-free material was separated by two step centrifugation and filtration. MV was quantified with flow cytometry and with protein determination. MV were investigated with electron and fluorescent microscopy, flow cytometry, light scattering, proteomics analysis and Western blotting. Biological effect was tested in bacterial killing assay.
Results: Production of MV was observed upon stimulation with chemoattractants or phorbol ester (C-MV). Significantly higher amount of MV was detected after incubation with opsonized *S. aureus* (B-MV). Formation of B-MV depended on intact cytoskeleton of PMN and on opsonisation of bacteria, while it was inhabitable with azide or di-phenyl-iodonium.

MV are heterogenously sized (d = 200–1000 nm) vesicles surrounded by phospholipid membrane. They contain several granule enzymes, cytoskeletal proteins, but they are devoid of subunits of the NADPH oxidase and nucleic acids. Proteomics analysis revealed differences between C- and B-MV in neutrophil defensin 3, myeloperoxidase, integrin II B, lactotransferrin.

In bacterial killing assay C-MV had no effect on bacterial survival, while B-MV was able to impair bacterial growth, independently of opsonisation. Remarkable clamping of bacteria was observed with B-MV, while C-MV showed no clamping.

Conclusion: PMN-derived MV may represent a new extracellular mechanism that limits bacterial growth.

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Escherichia coli K-12 (luxABCDEamp). A tool for analysis of bacterial killing by complement and myeloperoxidase activities in real-time basis

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Background: Our aim was to construct a tool for analysis of bacterial killing by complement and myeloperoxidase (MPO) activities on a real-time basis.

Materials and methods: We made the recombinant strain of *Escherichia coli* K-12 by cloning with luxABCDEamp gene expressing bacterioluciferase enzyme. Expression of whole luciferase operon produces cells capable of emitting bioluminescence (BL) without any addition of substrate. BL was measured using multilabel plate readers.

Results: We showed that the level of BL was proportional to amount of viable cells by incubating this microbe in wells of a test plate with serum and MPO and measuring the diminishing BL signal in short time intervals and achieving the kinetic curve of killing. BL signal was gained from the number of bacteria ranging from 10^7 to 10^2 live bacteria/well representing 99 999% of killing. The killing kinetics was undergoing exponential decay. From the kinetic curves several parameters for complement and MPO activities could be calculated. These parameters include length of a lag-phase where the activation of complement cascade from C1 to C5 occurs, time of 50%, 90%, 99%, etc, of killing and K_m and V_{max} for MPO.

Conclusions: The real-time approach is extremely elegant and provides a valuable tool to researchers whising to elucidate mechanisms of bacterial cell death in response to different antimicrobials. Compared to conventional plate counting our assay provides more accurate estimation of the bacterial viability with less labor.

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Gender-related differences with ageing in plasma levels of interleukin-6 and its soluble receptor as well as in the release of this cytokine by blood leukocytes

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Background: Males have worse cell immune response and higher inflammation state than females, showing a shorter lifespan than the last. Interleukin-6 (IL-6) is a cytokine involved in several relevant functions of leukocytes, but an increase in its plasma levels has been related with fragility and mortality risk. The aim of this work was to determine plasma levels of IL-6 and its soluble receptor (sIL-6R) as well as the IL-6 release by blood leukocytes (stimulated by lypopolysaccharide (LPS) and non-stimulated) in men and women of different ages.

Materials and methods: A total of 206 healthy human volunteers were studied, 103 women and 103 men. They were divided in three age groups: adult (30–49 years old), adult-mature (50–59 years old) and mature (60–79 years old). The levels of IL-6 and sIL-6R in plasma as well as of IL-6 in supernatants of LPS-stimulated and non-stimulated peripheral blood cells were measured by ELISA.

Results: The results showed higher values of plasma IL-6 levels ($P < 0{\cdot}05$) in men with respect to women due to the increase of its levels in mature men, as well as lower sIL-6R levels in men with respect to women ($P < 0{\cdot}05$). However, the percentage of IL-6 released in LPS-stimulated blood with respect to the non-stimulated samples decreased in men, especially in mature subjects ($P < 0{\cdot}05$).

Conclusions: In mature men the higher plasma levels of IL-6 and the lower release of this cytokine by blood leukocytes against an infection (LPS presence) could be one of the reasons of the higher mortality of men compared to females at that age.

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Age-related differences in the levels of tumour necrosis factor-alpha, its soluble receptors I and II, and interleukin-10 in men and women

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Background: With ageing an 'inflamm-ageing' occurs, which is more pronounced in men, due to the impairment of the immune system. The aim of this work was to check the inflammation state measuring the levels of a proinflammatory cytokine, tumour necrosis factor-alpha (TNF-α) and its soluble receptors (sTNF-RI and -RII), as well as an anti-inflammatory cytokine, interleukin-10 (IL-10), in plasma and supernatant of blood cells cultures.

Materials and methods: One hundred and fifty-three healthy human volunteers were studied, 76 women and 77 men. They were divided in three age groups: adult (30–49 years old), adult-mature (50–59 years old) and mature (60–79 years old). Levels of TNF-α, sTNF-RI, sTNF-RII and IL-10 were measured by ELISA in plasma and in supernatants of lypopolysaccharide (LPS)-stimulated peripheral blood cells.

Results: The results showed statistically significant increases of TNF-α in the mature subjects with respect to the adults ($P < 0{\cdot}05$) in supernatant of LPS-stimulated blood, especially in men. This age-related increase is associated with high plasma levels of sTNF-RI and -RII. Moreover, IL-10 levels in LPS-stimulated blood decrease with age in the mature subjects ($P < 0{\cdot}05$). No changes were observed with aging in plasma cytokine levels.

Conclusions: With ageing, there is an increase of TNF-α release and a decrease of IL-10 secretion by leukocytes in response to LPS. These results confirm the involvement of immune cells in the inflammatory stress of ageing, especially in men. In addition, the organism response increasing levels of TNF soluble receptors, trying to neutralize the proinflammation excess, is also shown.

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Anti-ApoA-1 auto-antibodies increase cardiovascular vulnerability

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Background: Anti-ApolipoproteinA-1 auto-antibodies (anti-ApoA-1 IgG) represent an emerging prognostic cardiovascular marker in patients with myocardial infarction or autoimmune diseases associated with high cardiovascular risk. The potential relationship between anti-ApoA-1 IgG and plaque vulnerability remains elusive. Thus, we aimed to investigate the role of anti-ApoA-1 IgG in plaque vulnerability.
Materials and methods: Potential relationship between anti-ApoA-1 IgG and features of cardiovascular vulnerability was explored both *in vivo* and *in vitro*. *In vivo*, we investigated anti-ApoA-1 IgG in patients with severe carotid stenosis ($n = 102$) and in ApoE−/− mice infused with polyclonal anti-ApoA-1 IgG. *In vitro*, anti-ApoA-1 IgG effects were assessed on human primary macrophages, monocytes and neutrophils.
Results: Intraplaque collagen was decreased, while neutrophil and MMP-9 content was increased in anti-ApoA-1 IgG positive patients and anti-ApoA-1 IgG-treated mice as compared to corresponding controls. In humans, serum anti-ApoA-1 IgG levels positively correlated with intraplaque macrophage, neutrophil and MMP-9 content, and inversely with collagen. *In vitro*, anti-ApoA-1 IgG increased macrophage release of CCL2, CXCL8 and MMP-9, as well as neutrophil migration towards TNF-α or CXCL8.
Conclusions: These results suggest that anti-ApoA-1 IgG might be considered as active factors that directly increase atherosclerotic plaque vulnerability in humans and mice.

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Leukocyte mitochondrial membrane potential in type 1 diabetes families

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Background: Proper cellular function requires the maintenance of mitochondrial membrane potential (MMP) sustained by the electron transport chain. Mitochondrial dysfunction is believed to play a role in the development of diabetes and diabetic complications possibly because of the active generation of free radicals. Since MMP can be investigated in clinical settings using fluorescent probes and living whole blood cells, mitochondrial membrane alterations have been observed in some chronic disorders.
Materials and methods: We have used the mitochondrial indicator 5,5′,6,6′-tetra chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) in conjunction with flow cytometry to measure the MMP in peripheral blood granulocytes from type 1 diabetes (T1D) families (diabetic probands and non-diabetic siblings). The intracellular ROS levels and the respiratory burst activity were also measured.
Results: Leukocyte MMP was elevated in 20 T1D patients and their 20 non-diabetic siblings compared with 25 healthy subjects without family history of T1D. Fasting plasma glucose was the only correlate of MMP.
Conclusions: The flow cytometric finding of mitochondrial hyperpolarisation in circulating leukocytes suggests a functional synchronization across the mitochondrial network in T1D family members, even without frank diabetes. The functional implications of mitochondrial hyperpolarisation (probably different among different cells) will require extensive investigation.

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Identification of formyl peptide receptor agonists through screening of a compound library

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Background: The formyl peptide receptor 2 (FPR2) is a G-protein coupled receptor that can induce pro-inflammatory as well as anti-inflammatory activities when it binds a specific agonist. Accordingly, this receptor has become a therapeutic target for the development of novel drugs that may be used to reduce inflammation.
Materials and methods: We used a molecular library high-throughput screening approach to identify small molecule FPR2 agonists with ability to induce a transient rise in intracellular Ca^{2+} in transfected cells expressing FPR2. The agonistic effect of identified molecules was further examined in neutrophil functional assays.
Results: Out of the 50 k small molecule library compounds, 10 gave rise to a calcium response in the FPR2 transfectants. All 10 compounds activated human neutrophils to produce/release superoxide anions, and based on the potency of their activity, the three most potent activators of the neutrophil NADPH-oxidase were further characterized. These three agonists induced chemotaxis, granule mobilization and secretion of reactive oxygen species (ROS). The neutrophil response was largely inhibited by cyclosporine H, an FPR1 selective antagonist, but not by PBP10, and FPR2 selective inhibitor, suggesting that FPR1 is the preferred receptor for all three small molecule agonists. We also show that these compounds are stable and resistant to oxidation.
Conclusions: Using a molecular library screening approach, we have identified novel and stable neutrophil FPR agonists and FPR1 is the preferred receptor.

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A role of the mammalian actin-binding protein 1 (mAbp1) for post-arrest spreading and intraluminal crawling of neutrophils under flow conditions

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Background: The mammalian actin-binding protein 1 (mAbp1) is required for β_2 integrin-mediated neutrophil adhesion under flow conditions. Here we studied the role of mAbp1 for spreading and migration of neutrophils.

Materials and methods: Murine neutrophils were isolated from the bone marrow of mAbp1$^{-/-}$ and mAbp1$^{+/+}$ mice. For fluorescence microscopy and co-immunoprecipitation experiments, neutrophil-like differentiated HL-60 cells (dHL-60) expressing mAbp1-EGFP were used. Mechanotactic migration was analyzed in flow chambers and chemotactic migration in Zigmond chambers.

Results: Confocal microscopy revealed that mAbp1 was enriched at the leading edge of polarized dHL-60 cells. Here mAbp1 colocalized with F-actin and Syk. The enrichment of mAbp1 at the lamellipodium was dependent on Syk as it was abrogated upon pharmacological inhibition of Syk. TIRF microscopy showed that mAbp1-EGFP formed highly dynamic structures at the lamellipodium. Accordingly, co-immunoprecipitation experiments revealed that β_2 integrin-mediated adhesion induced the interaction between mAbp1 and actin. Functional analysis showed that the genetic absence of mAbp1 had no effect on neutrophil spreading under static conditions. In contrast, mAbp1$^{-/-}$ neutrophils showed compromised spreading on fibrinogen and on intercellular adhesion molecule 1 (ICAM-1) under flow conditions. Similarly, chemotactic migration under static conditions was unaffected, but mechanotactic migration on ICAM-1 was severely compromised in mAbp1$^{-/-}$ PMN under flow conditions compared to control.

Conclusions: These findings suggest that mAbp1 is downstream of Syk during β_2 integrin-mediated signaling and concomitantly provides a functional link between the β_2 integrins and the cytoskeleton. This dual function of mAbp1 seems to be critical for the transmission of mechanical force from the actin cytoskeleton via β_2 integrins to extracellular ligands, which is necessary for intraluminal spreading and crawling of neutrophils during their recruitment to sites of inflammation.

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Different correlates suggest different pathogenic pathways for HIV associated minor neurocognitive impairment vs. HIV associated dementia

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Background: HIV-associated neurocognitive disorders (HAND) include minor disorders (MND/ANI) and dementia (HAD). HIV early invades the central nervous system (CNS) via monocytes. A persistent replication of HIV in perivascular macrophages and microglia is involved in HAND as are chronic inflammation of the CNS and possible neurotoxicity of long-term antiretroviral therapy.

Materials and methods: We measured monocyte receptor expression by 9-color flow cytometry and soluble factors by ELISA in blood and CSF samples from 20 HIV+ patients treated with combination anti-retroviral therapy (cART) who developed HAND.

Results: Blood monocyte percentage was surprisingly higher in patients with MND/ANI compared with HAD (P = 0·0017). This increase involved the CD14 + /CD16- subset MND/ANI could thus be the result of events occurring in the periphery. sCD163 level in the serum was inversely correlated with BAFF and MCP-1 levels in the CSF CSF lymphocytes, including mostly T lymphocytes, were CCR5-bright and CD69-bright. Of note, CXCL-12 concentration in the CSF correlated with CSF lymphocytes count, suggesting a role for this chemokine in lymphocyte attraction.

Conclusion: Our results suggest that common mechanisms govern both monocyte and lymphocyte migration from the periphery to the brain. CNS invasion by activated monocytes may alter the blood-CSF barrier and allow subsequent entry of lymphocytes. Finally, MND/ANI pathogenesis could be associated with blood specific feature whereas dementia rather relates with autonomic HIV replication.

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Decreased NADPH oxidase function due to mutations in *CYBB*. Diagnostic challenge in patients with X-linked chronic granulomatous disease

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Background: Chronic granulomatous disease (CGD) is an immune deficiency caused by defects in the leukocyte NADPH oxidase, due to mutations in any one of the genes encoding structural components of this enzyme. We studied NADPH oxidase capacity in two X-linked CGD patients with mutations in *CYBB* (encoding gp91^{phox}) that left gp91^{phox} protein expression intact but destroyed its activity.

Materials and methods: Both patients had recurrent, severe, pyogenic infections. Hydrogen peroxide and superoxide generation, bactericidal activity, NADPH oxidase component expression and genetic analysis were performed.

Results: We found that both patients have a novel missense mutation in *CYBB*, but intact gp91^{phox} expression. Surprisingly, the bactericidal activity was normal in one patient, although reduced at higher concentrations of bacteria. Further, neutrophils from both patients showed total absence of superoxide production, although they retained 13–30% of the hydrogen peroxide production capability. Since both mutations are in gp91^{phox} regions involved in docking of the cytosolic NADPH oxidase component p47^{phox}, we speculate that this is due to direct electron transfer from FAD in gp91^{phox} to oxygen, leading to inefficient hydrogen peroxide formation instead of efficient superoxide production.

Conclusions: X-linked CGD patients with mutations that alter gp91^{phox} in regions involved in docking of p47^{phox} may have disbalanced NADPH oxidase activity, affecting bactericidal activity.

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Development of a small-molecule inhibitor for P-Rex1

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Background: The small G protein Rac regulates reactive oxygen species (ROS) formation, phagocytosis, chemotaxis, spreading and adhesion in neutrophils, and can be activated by the guanine nucleotide exchange factor P-Rex1. In neutrophils, P-Rex1 controls fMLP-stimulated ROS formation, chemotaxis and adhesion, in cooperation with Vav1 (Lawson *et al*. J Immunol, in press). The aim of this project is to develop a small-molecule inhibitor for P-Rex1.
Materials and methods: An *in silico* screen of publically available small molecule libraries was used to identify compounds that could potentially bind to the catalytic DH domain of P-Rex1. A selection of these compounds was then tested for their ability to inhibit P-Rex activity *in vitro*.
Results: Of the 10 compounds identified by modelling to be the most likely to bind the P-Rex1/Rac interface, three inhibited P-Rex1-stimulated Rac2 activity with an IC_{50} of < 10 μM, with one inhibiting at an IC_{50} of 5 μM. In the absence of P-Rex1, neither the basal nor the EDTA-stimulated activities of Rac2 were affected by these compounds. The specificity of the inhibitors is currently being assessed, and we aim to use these lead compounds to identify analogues with the potential to inhibit at sub-micromolar concentrations.
Conclusions: Three compounds with potential inhibitory activity against P-Rex1 have been identified. These preliminary results will form the basis of further experiments with the goal of developing a small-molecule compound that can specifically inhibit P-Rex1 in neutrophil-mediated inflammatory disorders.

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Chikungunya virus infection involved monocytes and during chronic phase of the disease persisted in tissue macrophages

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Background: Chikungunya virus (CHIKV) has recently caused millions cases worldwide. This virus, beside acute phase arthralgia and myalgia, causes persistent rheumatic disease.
Materials and methods: We evaluated CHIKV disease (CHIKVD) in adult macaques by analysis of viral replication, immune response and cytokine expression during the acute and recovery phases of infection. We explored tissue modification and identified sites of viral replication using RT-PCR, immuno-histochemistry and *in situ* hybridization between 2 days and 6 months post-infection (pi).
Results: CHIKVD paralleled the human disease recapitulating fever, rash, arthritis and rare CHIKV lethal encephalopathy. We observed an increase in pro-inflammatory cytokines and chemokines, IL6, type I IFN, IFNgamma and MCP-1, accompanied by lymphopenia and monocytopenia as well as macrophage infiltration in tissue. CHIKV was detected in monocytes in acute infection then in tissue macrophages up to at least 3 months pi (Labadie K *et al.* J Clin Invest 2010). *In vitro*, macrophages but not monocyte-derived dendritic cells are susceptible to infection. In a new murine model of CHIKVD, the role of monocyte/macrophage lineage has also been evidenced (Gardner J *et al*. J Virol 2010).
Conclusions: Infectious CHIKV persists for extended periods in the macrophages in target tissues. Finally, we identified monocytes/macrophages as key players in the pathogenesis of CHIKVD in both acute and chronic phases of infection where they may be cellular reservoirs, potentially explaining long-lasting symptoms observed in humans.

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Involvement of anionic phospholipids in NADPH oxidase subunit recruitment during phagocytosis

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Background: During phagocytosis, neutrophils kill pathogens through the production of reactive oxygen species (ROS) by the NADPH oxidase (NOX2). Anionic phospholipids (phosphatidylserine (PS) and phosphoinositides) bind to cytosolic subunits p47 and Rac through electrostatic interactions. We investigated the role of PS in p47 and Rac phagosomal membrane recruitment.
Materials and methods: We used the C2 domain of lactadherin (lactC2) that interacts strongly and specifically with PS to monitor intracellular PS localization and to decrease PS accessibility. Differentiated PLB985 cells were transfected with mcherry-lactC2, p47-GFP, GFP-Rac2G12V, and their localisation during phagocytosis of zymosan was followed by videomicroscopy. The kinetics of phagosomal ROS production was measured with DCFH2-labelled yeasts.
Results: PS was present at the plasma membrane and at the phagosomal membrane during phagosome formation. However, 20 min after the phagosome closure, the PS content was reduced by half at the phagosomal membrane. A delay in the phagosomal ROS production was observed during the first 10 min in cells expressing mcherry-lactC2. This suggests that lactC2 masked PS and possibly disturbed NOX2 subunit attachment. We observed a transient localisation of p47 and constitutively active Rac2 at the phagosomal membrane during phagocytosis and studied the impact of lactC2 expression on their recruitment.
Conclusions: The results show that p47 and Rac2 are transiently recruited at the phagosomal membrane while ROS production continues for a longer period in a PS-dependent manner.

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Notch- and transducin-like enhancer of split (TLE)-dependent histone deacetylation explain IL-12 p70 inhibition by zymosan in human monocyte-derived dendritic cells

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Background: IL-12 p70/IL-23 balance is central to the development of the Th1 and Th17 responses. IL-12 p70 and IL-23 share a common chain and differ by another chain, IL-12 p35 (*il12a*) in IL-12 p70 and IL-12 p19 (*il23a*) in IL-23. The

molecular mechanism explaining the IL-12 p70/IL-23 balance relies on the regulation of *il12a* and *il23a*.

Materials and methods: Human monocyte-derived dendritic cells were obtained by differentiation with GM-CSF and IL-4. Phosphorylation of c-Rel and histone H3 by mitogen- and stress-activated kinase (MSK) and protein kinase A were studied in cells and in recombinant proteins. Chromatin immunoprecipitation was used to study the transcriptional regulation of *il12a* and *il23a*. Coimmunoprecipitation assays were used to address the association of the Notch family proteins with acetylated-K14-histone H3.

Results: The transcriptional regulation *of il23a* depended on the activation of c-Rel and histone H3 phosphorylation, as judged from the association of c-Rel with the *il23a* promoter and the correlation between IL-23 production and S10-histone H3 phosphorylation. Zymosan blocked the transcription of *il12a* induced by other stimuli and triggered the nuclear translocation of the transcriptional repressors Hes1, Hes5, Hey1, and TLE. In addition, zymosan induced the interaction of Hes1 and TLE with histone H3 phosphorylated on S10 and deacetylated on K14. Inhibition of class III histone deacetylases increased the production of IL-12 p70 and partially blunted the inhibitory effect of zymosan on the production of IL-12 p70.

Conclusions: The selective induction of IL-23 by β-glucans is explained by the activation of c-Rel associated with S10-histone H3 phosphorylation in the *il23a* promoter and inhibition of *il12a* transcription by a mechanism involving corepressors with ability to bind TLE and to promote histone deacetylation.

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The guanine-nucleotide exchange factor P-Rex1 is activated by protein phosphatase 1α

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Background: P-Rex1 is a guanine-nucleotide exchange factor (GEF) for the small G protein Rac that controls GPCR-dependent ROS formation in neutrophils and is important for neutrophil recruitment to sites of inflammation. P-Rex1 is activated by PIP_3, a lipid second messenger generated by PI3K, and by the Gβγ subunits of heterotrimeric G proteins. P-Rex1 is inhibited by the protein kinase PKA. Here, we have investigated the possibility of P-Rex1 regulation by dephosphorylation.

Materials and methods: Protein binding assays *in vivo* and *in vitro*. Rac-GEF activity assays *in vivo* and *in vitro*. Cell morphology assays. Mass spectrometry. Mutagenesis.

Results: We show that Protein Phosphatase 1α (PP1α) directly binds P-Rex1 through an RVxF-type docking motif. PP1α activates P-Rex1 directly *in vitro*, both independently of and additively to PIP_3 and Gβγ. PP1α also substantially activates P-Rex1 *in vivo*, both in basal cells and in response to PDGF or LPA stimulation. The phosphatase activity of PP1α is required for P-Rex1 activation. PP1β, a close homologue of PP1α, is also able to activate P-Rex1 but less effective. PP1α stimulates P-Rex1-mediated, Rac-dependent changes in endothelial cell morphology. Mass spectrometric analysis of wild type P-Rex1 and a PP1α-binding deficient mutant revealed that endogenous PP1α dephosphorylates P-Rex1 on at least three residues, S834, S1001 and S1165. Site-directed mutagenesis of S1165 to alanine activated P-Rex1 to a similar degree as did PP1α, confirming S1165 as a dephosphorylation site important in regulating P-Rex1 Rac-GEF activity.

Conclusions: In summary, we have identified a novel mechanism for direct activation of P-Rex1 through PP1α-dependent dephosphorylation.

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A founder effect for p47 Trp193X CGD?

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Background: Mutations in *NCF1* lead to p47-*phox*-deficient CGD. Most of these mutations are due to unequal crossing over with one of the pseudo-*NCF1* genes, but we found 17 families (nine from a Jewish subpopulation until recently residing in the Caucasus, eight from Mediterranean countries) with a c.579G>A mutation, predicting p.Trp193X. We have investigated whether these families can be traced back to one common ancestor.

Materials and methods: We analyzed two SNPs within *NCF1*, determined mtDNA markers and investigated two short tandem repeats (STRs) within the *NCF1* gene cluster (chromosome 7). We included three control Jewish population groups from the Caucasus area, three Caucasian and Mediterranean non-Jewish groups and one West-European group.

Results: The c.579G>A mutation showed 100% linkage with c.295A/G and c.345C/T SNPs within *NCF1* (all patients GG and TT, respectively; all normals AG or AA and CT or CC, respectively). The mtDNA markers showed considerable heterogeneity within and between Jewish and non-Jewish patients. STR analysis showed a shared haplotype within the Jewish patients, whereas some of the non-Jewish patients had the same haplotype while others had related haplotypes. This was true for both STRs tested.

Conclusions: Most probably, the c.579G>A mutation in *NCF1* originates from one mutational event in the Mediterranean area, that was later introduced into a Jewish subpopulation.

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Regulation of complement receptor Ig by pro-inflammatory and anti-inflammatory agents: implications in infection and immunity

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Background: CRIg, a high affinity complement receptor, is expressed on interstitial macrophages. Kupffer cell surface CRIg leads to a rapid sequestration of circulating *Listeria monocytogenes* and *Staphylococcus aureus*, thereby limiting bacterial dissemination and pathogenesis, excessive innate and adaptive immune system activation, exaggerated levels of inflammatory cytokines and mortality. While the role of CRIg in protection against infection and microbial pathogenesis has been established, little is know on the regulation of its production in macrophages. Since during the inflammatory reaction cellular activation leads to the release of arachidonate, which stimulates leukocyte function, it was of interest to determine if arachidonate regulates CRIg expression.

Materials and methods: Reverse transcriptase polymerase chain reaction and fluorescent activated cell sorter were used to detect human CRIg mRNA and protein levels, respectively.

Results: Cell surface CRIg and CRIg mRNA expressed in maturing human macrophages as well as in pre-matured CRIg$^+$ macrophages was significantly decreased by addition of arachidonate, in a similar manner to the addition of Th1 cytokines such as interferon-γ. The effect was independent of the metabolism of arachidonate via the cyclooxygenase and lipoxygenase pathway, since the non-steroidal anti-inflammatory agents indomethacin and nordihydroguaiaretic acid did inhibit its expression. Using specific pharmacological inhibitors of the intracellular signalling pathways activated by arachidonate, protein kinase C was found to be involved in this effect. In contrast, dexamethasone caused a marked up-regulation of CRIg expression in macrophages.
Conclusions: Arachidonate regulates CRIg expression in macrophages in a PKC-dependent manner. Thus, the ability of microbial pathogens to directly release arachidonate and stimulate its release from host cells is likely to hinder the initial 'silenced jailing' of pathogens by CRIg and promoting microbial dissemination and pathogenesis.

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Heat-treatment reduces protein degradation in the neutrophil proteome

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Background: As the main cells of the innate immune system, neutrophils contain an array of proteases and reactive oxygen species to control the invasion of bacteria and pathogens. The quantity of intracellular proteolytic enzymes makes it a difficult cell to work with as they can degrade proteins of potential interest. Here we describe the benefits of heat-treatment of neutrophils in reducing protein degradation for subsequent proteome analysis.
Materials and methods: Neutrophil isolates from four volunteers were each divided into three aliquots and subjected to different preparation methods for 2-Dimensional Electrophoresis (2-DE); (i) Heat-treated, (ii) Resuspended in NP40 lysis buffer, (iii) Resuspended in standard 2-DE lysis buffer. Differences in the resulting 2-DE protein spot profiles determined by Progenesis SameSpots software were excised and identified by liquid chromatography tandem mass spectrometry (LC-MS/MS).
Results: Samples resuspended in standard 2-DE lysis buffer displayed such an extent of sample degradation they could not be aligned with NP40 and heat-treated samples for image analysis. Heat-treated samples contained proteins in the high molecular weight range that were absent from NP40 treated samples (which included Gelsolin, (verified by Western blot), Serpin B1 and Protein Disulfide Isomerase). Moreover, NP40 treated samples showed an increase in spot number and volume at lower molecular weights suggestive of protein degradation.
Conclusion: Incorporating heat-treatment into sample preparation resulted in the identification of proteins that may not have previously been detected due to sample degradation. This will lead to a definitive map of the human neutrophil proteome which may contribute to an increased understanding of the cellular processes involved in neutrophil activation and signalling.

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Protective effect of glycoconjugates in iron deposition in human *globus pallidus*

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Background: The iron, it is one of the biogenic elements that play an essential physiological role in all organisms. In the human brain, iron plays a decisive role in the process of aging and neurodegenerative diseases. The reason of iron deposition in human brain is still unknown. The aim of the study is investigation of distribution of acidic and neutral glycoconjugates deposits in human *globus pallidus* and its relation to nonheme ferric Fe(III).
Materials and methods: A total of 18 formalin-fixed and paraffin-embedded human necropsy tissue specimens with *globus pallidus* of normal brain, without any motor abnormalities and psychiatric symptoms in patient during life, were besides conventional histological stains evaluated by histochemistry with using Prussian blue reaction, Alcian blue, PAS reaction, Perls' method, von Kossa reaction and Alizarin red reaction, by light microscopy, for detection a nonheme ferric Fe(III) and any other chemical substances presence and distribution in brain tissue.
Results: We found population of dark blue cells and dark blue granular deposits around glial cells and in the *tunica media* of small blood vessels after Perls' staining for iron detection. Both von Kossa reaction for phosphate detection and Alizarin red reaction for calcium detection were very faintly positive. We detected acidic glycoconjuagtes (Alcian blue) and neutral glycoconjuagtes (PAS reaction) deposits with the darker centre (glial cells) and in the *tunica media* of small blood vessels. These deposits colocalize with iron deposits.
Conclusions: The occurrence of iron deposits in the basal ganglia appears to be age-related. We suppose the presence of glycoconjugates in human *globus pallidus* is result of elimination and inactivation of iron as an inductor of reactive oxygen species and can be useful in preventing of CNS degradation.

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L-arginine is critically involved in the regulation of macrophage physiological functions

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Background: L-arginine was shown to exert the pleiotropic effects on different cell populations including macrophages which are mediated by a number of factors that are still mostly undefined.

Methods: Murine leukaemic macrophage cell line RAW 264·7 was used for experiments. Cells were cultured in L-arginine-free media or in the media with different L-arginine concentrations (100, 200, 300 and 400 μM). Intracellular L-arginine was determined using a LC-MS/MS. Chemotaxis was assessed by μ-Slide® system. Oxidative burst and phagocytosis were measured using luminimetric and flow cytometric analysis. The activity of NF-κB was determined by luciferase activity measurement. Release of cytokines and chemokines was tested using cytokine array. Nitrite accumulation was determined by Griess reagent and expression of iNOS protein and MAP kinases was determined using Western blot.
Results: L-arginine caused a significant and dose-dependent increase in proliferation, migration and production of several cytokines in nonstimulated macrophages. Moreover, it enhanced the oxidative burst, phagocytosis, production of NO and proinflammatory cytokines in endotoxin-stimulated macrophages. Detailed analysis showed that L-arginine is crutial for the activation of MAP kinases and NF-κB that is followed by regulation of iNOS expression.
Conclusion: Our data clearly demonstrated the importance of L-arginine in the regulation of macrophage physiological functions which is probably connected with the activation of G-protein coupled receptors.

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Characterization of a new signaling pathway involved in the regulation of the neutrophil NADPH oxsidase through S100A8/A9 translocation

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Background: The neutrophil NADPH oxidase (NOX2), through the production of reactive oxygen species, is a key enzyme for host defense against invading pathogens. Although, it is established that the translocation of S100A8/S100A9, two Ca^{2+}-binding proteins, is involved in NOX2 regulation, mechanisms underlining such a process remains elusive.
Materials and methods: Neutrophil-like HL-60 cells and human neutrophil were subjected to pharmacological inhibitors or transfected by specific siRNA. NOX2 activity and intracellular Ca^{2+} variations were quantified by spectrofluorimetry using Amplex red and Fura-2/AM for H_2O_2 and Ca^{2+} measurements. S100A8/A9 translocation was monitored by immunofluorescent labeling with Mac387 antibody. p38 MAPK activity was performed using a p38 MAPK activity test (Cell Signaling). Finally, protein-protein interactions were characterized by GST pull-down.
Results: Our data show that sphingosine kinases (SphK) are involved in the S100A8/A9 recruitement to the plasma membrane. Depletion of internal Ca^{2+} stores is required to mediate SphK activity dependent-S100A8/A9 translocation. Further, SphK knock-down resulted in a decrease of p38 MAPK activity. In addition, we observed that inhibition of S100A8/A9 translocation by SphK knock-down is associated to a decrease of NOX2 activation. S100A8/A9 interacts with cytosolic subunits of NOX2 ($p67^{phox}$, $p47^{phox}$, Rac1 and Rac2). These interactions are not inhibited by intracellular Ca^{2+} chelation.
Conclusions: Ca^{2+} store depletion-induced SphK activity regulates NOX2 activity through the p38 MAPK dependent-translocation of S100A8/A9. Moreover, S100A8/A9 interacts with NOX2 cytosolic factors in a Ca^{2+} independent manner suggesting that S100A8/A9 regulates NOX2 assembly to the plasma membrane.

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Proinflammatory stimuli enhance clearance of apoptotic cells by neutrophil granulocytes

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Background: Clearance of apoptotic neutrophils is essential to prevent uncontrolled inflammation. It is generally believed that macrophages and dendritic cells in inflamed tissues clear those apoptotic cells. Recently our group found that also neutrophil granulocytes can phagocytose apoptotic neutrophils in a serum dependent manner [Esmann et al., J Immunol. 184:391, 2010]. Based on this finding we hypothesized that 'cannibalistic' neutrophils at sites of acute infection/inflammation play a major role in the clearance of apoptotic neutrophils.
Materials and methods: In the present study we analyzed the effect of microbial constituents and proinflammatory cytokines present at sites of acute inflammation on the ability of neutrophils to phagocytose apoptotic cells. Phagocytosis was analyzed by flow cytometry in a system containing only neutrophils or in full blood.
Results: We were able to show that exposure to ligands of TLR2 (Malp2, Pam3Cys-SKKKK), TLR4 (LPS), TLR7/TLR8 (Imiquimod, R848) and TLR9 (ODN 2006) led to enhanced phagocytosis of apoptotic cells by neutrophils. In addition, proinflammatory cytokines that can be present in acutely inflamed tissues, such as TNF and GM-CSF, strongly enhanced the uptake of apoptotic cells by neutrophils.
Conclusions: Taken together these results are in line with our hypothesis that neutrophils acquire the ability to phagocytose apoptotic cells at sites of acute infection/inflammation. Since uptake of apoptotic cells is clearly anti-inflammatory, enhanced clearance of apoptotic cells by neutrophils is likely to contribute to the resolution of local inflammation.

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Highly pathogenic influenza viruses cause an inhibition of the immune response in human monocytes via activation of the Rar-related orphan receptor alpha

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Background: A characteristic symptom of infection with highly pathogenic avian influenza viruses (HPAIV) is the so-called cytokine storm. Considering monocytes to be possible initiators of the cytokine storm, we analyzed signalling pathways induced in influenza infected human monocytes.
Materials and methods: Primary human monocytes were infected with H5N1 virus as well as with two lower pathogenic virus strains, FPV and PR8. The cell response was analyzed by qRT-PCR, FACS staining, Western Blot and Affymetrix gene array for three independent blood donors. Heterozygous RORα knockout mice were purchased from Jackson Laboratories.

Results: Microarray analysis as well as qRT-PCR experiments revealed that HPAIV cause a suppression of the immune response in human monocytes. Performing Carrie analysis we were able to identify transcription factor binding sites overrepresented in influenza virus induced genes. Interestingly, binding sites for NF-κB could not be detected and NF-κB target genes known to play a role during influenza infection of other cell types were not induced. We could show by qRT-PCR and Western Blot that the mechanism behind these observations is an inhibition of the NF-κB signalling pathway by the immunosuppressive transcription factor RORα for which an overrepresentation of binding sites could be shown. These results could be confirmed by experiments with heterozygous RORα knockout mice which showed a stronger immune response to influenza infection than wildtype littermates.
Conclusions: We present for the first time a role of the transcription factor RORα during influenza infection. Activation of RORα affects the NF-κB signalling pathway and possibly further signalling cascades. A reduction of RORα activity leads to a stronger immune response to influenza infection in mice. This emphasizes the physiological relevance of our findings.

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Primary human blood-derived macrophages restrict release of infectious viral particles

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Background: Infections with highly pathogenic avian influenza viruses (HPAIV) like H5N1 cause systemic spreading of infection and a strong release of cytokines. The detailed mechanism leading to viral distribution within the body and to the emergence of a cytokine storm is still unknown. We investigated the role of human blood-derived macrophages in influenza infection and their contribution to cytokine release and viral replication.
Materials and methods: Primary human macrophages were infected with HPAIVs H5N1 and FPV as well as with a lower pathogenic virus strain, PR8. The cell response was analyzed by qRT-PCR, FACS staining, Western Blot and Affymetrix gene array for three independent blood donors. Virus replication was examined by plaqueassay, HA-test and immunofluorescence.
Results: A genome-wide systems biology approach revealed a reduced immune response after infection with H5N1 and FPV in comparison to infection with PR8. qRT-PCR experiments confirmed these findings. Replication of viruses within the cell could be detected by immunofluorescence staining of the viral nucleoprotein NP. However, we could not detect any infectious particles in supernatants of infected macrophages and in the case of H5N1 infection we could not even find incomplete viruses released from the cells. As a reason for this restriction of virus release we found an inhibition of Influenza A virus M2 protein translation. This protein is important for virion-building and -release.
Conclusions: We were able to find a novel mechanism by which human macrophages prevent viruses from complete replication. However, we could also reveal a viral strategy to bypass a major amplifier of the initial inflammatory response which may hamper antiviral effector mechanisms of other cell types resulting in virus spreading and systemic disease.

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AdIL-10-transduced bone marrow-derived macrophages protects from renal ischemia via iron and Lipocalin-2

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Background: Ischemia-reperfusion injury is a leading cause of acute renal failure and triggers an inflammatory response associated to infiltrating macrophages which determines the outcome of disease.
Material and methods: By means of the adoptive transfer of predominantly anti-inflammatory macrophages over-expressing IL-10, we designed an effective cellular therapy for preventing ischemic renal injury.
Results: AdIL-10-transduced bone marrow-derived macrophages were able to increase intracellular iron content which in turn increased Lipocalin-2, megalin, and Lipocalin-2 receptor expression. Macrophages infused in rats 1 h after the induction of the reperfusion localized efficiently to inflamed/injured kidney tissue. The delivery of IL-10-expressing macrophages caused increases in the regenerative markers Ki-67 and PCNA and marked reductions in both BUN and creatinine levels compared with the unmodified ischemia/reperfusion group. Furthermore IL-10 treatment decreased the local inflammatory mediator profile and up-regulated the expression of pro-regenerative Lipocalin-2 and its receptors *in vivo*. Interestingly, IL-10 mediated protection and subsequent renal regeneration were clearly dependent on the presence of iron and Lipocalin-2 *in vivo*, since administration of a neutralizing antibody for Lipocalin-2 or administration of IL-10-macrophages previously subjected to the iron chelating agent deferoxamine (DFO) abrogated IL-10-mediated protective effects.
Conclusion: In this study we describe an effective macrophage therapy mediated through the action of iron as a Lipocalin-2 enhancer, which in turn acts as a mediator of the anti-inflammatory and beneficial effects of IL-10 therapy.

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The impact of various reactive oxygen species on the formation of neutrophil extracellular traps

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Background: Activated neutrophils are able to produce complex three-dimensional structures, so called neutrophil extracellular traps (NETs), which are composed of chromatin, histones and granule proteins. Their function is to bind and kill microbes independently of phagocytotic uptake. The release of NETs by neutrophils is called NETosis, an active form of cell-death differing from apoptosis and necrosis. NETosis depends on the production of reactive oxygen species (ROS). However, it is speculated that ROS act as second messengers, but the involved ROS species and the nature of downstream signalling are still unknown.
Materials and methods: We have investigated which ROS species are involved in NETosis *in vitro*. In our present study

enzymes and mediators responsible for ROS formation were inhibited by using specific inhibitors. The production of H_2O_2, OCl^- and $O_2^{\bullet-}$ and the release of NETs were assessed. To induce the formation of ROS and NETs, freshly isolated human neutrophils were stimulated with PMA.

Results: Inhibition of NOX2 by the specific inhibitor DPI led to a total inhibition of the generation of H_2O_2 and $O_2^{\bullet-}$. In addition, significant reduction of H_2O_2, OCl^- was observed if the superoxide dismutase and myeloperoxidase were inhibited accordingly with Aroclor or Antipyrine. Mitochondrial superoxide production was down-regulated by inhibitors like rotenone and 2.4 Dinitrophenol.

Conclusions: The NET formation is dependent on NOX2-generated superoxide and on ROS species (H_2O_2, OCl^-) generated by superoxide dismutase and myeloperoxidase. Mitochondrial superoxide production has less influence on the formation of NETs.

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Time -dependent expression of cell surface antigens during the differentiation of HL-60 and PLB-985 myeloid cells into mature granulocytes

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Background: Neutrophil surface molecules function in part as biological sensors. Surface antigens undergo several changes during myeloid differentiation to accommodate cell functions. Here we describe time-dependent changes in antigen expression at the surface of HL-60 myeloid cells when differentiation is induced into granulocytes by dimethylsulphoxide (DMSO), all-trans retinoic acid (ATRA), as well as PLB-985 myeloid cells induced to differentiate into neutrophils by a mixture of *N,N*-Dimethylformamide (DMF) and Nutridoma-SP.

Material and methods: Terminally differentiated cells were compared to circulating neutrophils in terms of cell surface antigen expression by flow cytometry analysis using a panel of pertinently purported 35 antibodies.

Results: The changes in myeloid cell surface antigens induced by DMSO, ATRA or DMF/Nutridoma-SP paralleled the expression pattern of these molecules in normal granulopoeisis with the exception of seven antigens up-regulated and nine down-regulated, indicating that the maturation was most probably not achieved and thus partially defective. All these differentiation inducers failed to induce the expression of neutrophil specific markers CD16 and CD66b. Differentiated PLB-985 cells appeared closer to neutrophils, in term of maturation and CD expression than the DMSO- or ATRA-differentiated HL-60. Finally, single cell analysis of the expression dynamics of several differentiation markers (CD11b, CD14, CD35, CD71) revealed a bistable switch and not a graded change of expression when measured as a cell population average.

Conclusions: These results demonstrate kinetic changes in cell surface antigen expression during the transformation of a proliferating leukaemic cell into a potentially mature and terminally differentiated cell, which might be of substantial importance for analyzing the gene expression pathways that govern granulopoiesis.

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Sphingosine-1-phosphate signalling induced proliferation by activating anti-inflammatory macrophage phenotype through lipocalin-2 production

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Background: Macrophage influx after acute injury is essential to promote repair, also in complex tissues as kidney. Here, we explore the factors by which macrophage induce epithelial repair following ischemia. We establish that the mechanism involves the production and secretion of the protein lipocalin-2 (Lcn2) by macrophages as response to S1P signaling, displaying anti-inflammation.

Material and methods: Male swiss mice were subjected to renal ischemia for 45 min and up to 48 h of reperfusion with/out administration of S1P agonist FTY720 (EC_{50} 1 mg kg^{-1}) combined with macrophage depletion or transfer and Lcn2 manipulation. Parameters of regeneration, proliferation, and localization were done.

Results: Activation of S1P signalling and the elevating production of Lcn-2 production from macrophages *in vivo* were required for efficient tissue repair. Utilizing an *in vitro* model of macrophage co-culture and cell arrest of tubular renal epithelial cells (NRK-52e), we obtained evidence of apoptotic cells as a source for S1P that triggers Lcn-2 release from macrophages due to coupling to S1P receptor 3 (S1P3). Besides proliferation, S1P as well as Lcn-2 suppressed pro-inflammation *in vitro* and *in vivo*, contributing to regeneration.

Conclusion: Overall, our data indicate that a macrophage-dependent S1P-S1P3-Lcn-2 axis may be beneficial for restoration of tissue function following an acute injury insult.

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Laminin expression in cell basement membrane of normal, hyperplastic and neoplastic endometrium

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Background: Cell basement membrane (BM) is highly specialized extracellular matrix structure, which plays an important role in anchoring epithelial cells and separating them from the adjacent stroma. BM is composed of several glycoproteins also, of which laminin, large multifunctional heterotrimeric glycoprotein, is the most abundant. Laminins have been found to

promote cell adhesion, migration, protease activity, proliferation, tumor growth, angiogenesis and metastasis. The study evaluates an association between morphological appearance of normal, hyperplastic and neoplastic endometrium, and between the degree of laminin expression in BM and of clinical findings.

Material and methods: Using immunohistochemistry we investigated the laminin expression in BM of endometrial epithelial cells from a total of 35 archived formalin-fixed and paraffin-embedded human biopsy (hysterectomy and curettage) tissue specimens with normal proliferative endometrium and compared it to the expression pattern in hyperplastic and neoplastic endometrium. The findings were evaluated by light microscopy semiquantitatively and related to clinical course.

Results: The laminin expression was high in normal proliferative and in hyperplastic endometrium, but it was gradually going down with the grade of histological differentiation of endometrioid histological subtype of endometrial carcinoma (less aggressive phenotype). In histological subtypes of endometrial carcinoma with aggressive phenotype the laminin expression was the lowest and it correlated with the worse clinical course of oncological illness.

Conclusions: There is high expression of laminin in normal and hyperplastic endometrium. Malignant changes of endometrium are accompanied by a decrease in laminin expression in cell BM. Altered composition and assembly of BM may influence carcinoma cell growth and invasion. Its evaluation by immunohistochemistry is a relatively cheap and simple method usable for clinical practice.

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Defects in neutrophil degranulation and bacterial killing in familial hemophagocytic lymphohistiocytosis type 5 caused by mutations in STXBP2

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Background: Familial hemophagocytic lymphohistiocytosis (FHL) is a rare condition characterized by genetic defects in CTL and NK cell degranulation and cytotoxicity, which may accumulate into a life-threatening condition known as macrophage activation syndrome. FHL can be caused by mutations in a number of different genes, including perforin (FHL2), Munc13-4 (FHL3) and syntaxin-11 (FHL4). Recently, the granule-associated docking protein syntaxin binding protein-2 (STXBP2, also known as Munc18-2) was identified as the disease-causing gene in FHL5 patients. Here, we report our studies on an FHL5 patient and establish a role of STXBP2 in neutrophil degranulation and bacterial killing.

Materials and methods: The FHL5 patient was 5 week old girl with a homozygous mutation c.1621G>A (p.Gly541Ser) in exon 18 of the STXBP2. A variety of functional assays were performed by using isolated periphoral neutrophil granulocyte from the FHL5 patient and controls.

Results: Exocytosis of the various neutrophil granules and the release of serine proteases were defective. Furthermore, cytotoxicity towards *E. coli* was significantly impaired, but no difference in *S. aureus* killing was observed.

Conclusions: Our findings establish defects in neutrophil degranulation and bacterial killing in FHL5 and this may provide an explanation for the increased susceptibility to gastrointestinal bacterial infection observed in these patients.

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Vesicle amine transport protein-1 (VAT-1) recruitment to human neutrophil membranes is dependent on phospholipase D

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Background: Phospholipase D (PLD) is a key enzyme in the production of phosphatidic acid (PA). PA has been reported to stimulate secretion, kinases and the activity of NADPH oxidase. However PA-binding proteins that regulate these functions are still poorly characterized in human neutrophils.

Materials and methods: Using phospholipid vesicles and mass spectrometry analysis we attempted to identify PA-binding proteins in neutrophil cytosol.The role of PLD in regulating their subcellular localization in cells was investigated following cell fractionation and fluorescence microscopy.

Results: We have identified eight putative PA-binding proteins including vesicle amine transport protein-1 (VAT-1), Rac2, Cdc42, RhoG and annexin III in neutrophil cytosol. Focusing then on VAT-1 characterization, we found that *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) induced a translocation of VAT-1 to neutrophil membranes. This recruitment was inhibited in the presence of a Src kinase inhibitor (PP2), and a selective PLD inhibitor (FIPI), showing that fMLF-induced PLD activity is important for the association of VAT-1 with membranes. PMA also induced VAT-1 translocation through a pathway dependent on PKC and PLD. Phosphorylation of VAT-1 was induced by fMLF and PMA but only the latter was dependent on PKC. Subcellular fractionation assessed the localization of VAT-1 in cytosolic, primary, secondary and tertiary granules and in plasma membrane compartments in resting neutrophils. After fMLF stimulation, VAT-1 was redistributed in tertiary granules and in plasma membrane compartments. PLD1 partially overlapped with the VAT-1 fraction in resting and fMLF-stimulated neutrophils. Confocal microscopy showed that VAT-1 has an intracytoplasmic granular pattern. Colocalization of VAT-1 with lactoferrin was partial and not enhanced after fMLF stimulation.

Conclusions: Our data suggest that PLD-derived PA regulates the association of VAT-1 with various neutrophil granules.

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Ca^{2+} events in apoptosis and necrosis of neutrophils

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Background: During apoptosis, neutrophils switch-off their responsiveness to stimuli. It is not known how progress to apoptosis and signalling shutdown are controlled and related. This study therefore aims to unravel the interconnections between the two processes.

Materials and methods: Neutrophils were isolated from venous whole blood. Fluo4-AM was used as a indicator for Ca^{2+} concentration, and AnnexinV-Cy3/FITC and Propidium Iodide were used as an indicator of PS externalisation and

necrosis respectively. Caged IP3 was released by UV exposure during visualisation.
Results: Although PS externalisation and nuclear morphology changes wee not correlated, PS externalised cells had an elevated cytosolic free Ca^{2+} concentration. Elevating cytosolic IP_3 (by photolytic uncaging of caged IP_3), to trigger the release of Ca^{2+} from stores and open Ca^{2+} influx channels, had no effect in the PS-externalised sub-population, suggesting that channels may be partially open. Blockade of the Ca^{2+} influx channels by SKF96365 and Ni^{2+} in aging neutrophils (> 24 h) reduced necrosis resulting in a larger number of cells being held at the PS-externalised stage. A requirement of Ca^{2+} for the transition to necrosis in PS externalised cells was also shown by reducing extracellular Ca^{2+} (1 mM EGTA) resulting in a reduced rate of necrosis by PS externalised neutrophils. Elevating cytosolic Ca^{2+} experimentally using ionomycin and high media Ca^{2+} resulted in increased necrosis, an effect reduced by calpain inhibition.
Conclusion: This data points to a role for elevated Ca^{2+} and calpain activation in the necrosis of neutrophils after nuclear morphology changes and PS externalisation during apoptosis.

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The role of calpain translocation in neutrophil spreading

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Background: Neutrophils spread and phagocytose by expanding their plasma membrane. μ-calpain is a Ca^{2+} activated cytosolic protease which contains a 'C2-like' domain and cleaves cytoskeletal elements which, in resting neutrophils, maintain wrinkles in the plasma membrane. This report gives evidence for calpain and C2domain translocates during neutrophil spreading and phagocytosis.
Materials and methods: Immunofluorescence from stained neutrophils, and myeloid and macrophage cell lines transfected with fluorescently tagged calpain and C2 domains (PKC) allowed fixed and real-time sualisation of translocation during cytosolic calcium increase and phagocytosis using confocal microscopy.
Results: Immunofluorescent staining of neutrophils showed that calpain is located at the plasma membrane in spread neutrophils and around the phagocytic cup in neutrophils during phagocytosis. Increasing the cytosolic Ca^{2+} concentration in cell lines expressing a fluorescently tagged classical PKC C2 domain showed that C2 like' domain of μ-calpain would allow it to translocate from the cytosol to the membrane when cytosolic calcium is increased in the neutrophil during spreading and phagocytosis.
Conclusion: This work has therefore shown that C2-domain and μ-calpain translocates *n* to the plasma membrane during neutrophil shape change which accompanies phagocytosis and cell spreading. Calpain-1 translocation may be a drug target for the control of neutrophil infiltration in inflammation.

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Ezrin and talin relocates from the plasma membrane to cytosol during neutrophil extravasation

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Background: All inflammatory events are mark by infiltration by leukocytes including neutrophils, which cross the endothelium before following migratory cues to the site of infection, and phagocytose infecting microorganisms. Before crossing the endothelial wall, the neutrophils spread on the endothelium. It has been proposed that the necessary additional membrane for this, results from unfolding of wrinkled cell membrane held in place by molecule like ezrin and talin, which are targets for cleavage by the Ca^{2+} activated protease, calpain-1. This reports that ezrin and talin detaches from the plasma membrane during neutrophil extravasation.
Materials and methods: Neutrophils were isolated from venous blood and oral saliva, and ezrin and talin analysed by western blotting and immunocytochemistry.
Results: Ezrin and talin were located at the plasma membrane in resting and formylated peptide stimulated neutrophils. Approximately 67% of the detectable ezrin and 97% of talin was always at the cell periphery. Talin was polarused in chemotactic cells and was associated with actin and CD11b. However, after transmigration through endothelial monolayers *in vitro*, the detected ezrin and talin was mainly cytosolic. The same translocation was observed in oral neutrophils which had extravasated *in vivo*. Western blotting detected both intact portion (69 kDa) and cleaved (50 kDa) ezrin, the cleaved product being reduced by calpain inhibition. Only intact talin (247 kDa) was observed in these cells.
Conclusion: Relocation of ezrin and talin away from the cell periphery accompanied transendotheial migration both experimentally and physiologically.

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A case of Williams Beuren syndrome with p47^{phox} deficient chronic granulomatous disease

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Background: A 21-year-old Caucasian male with Williams Beuren syndrome (WBS), a genetic disorder characterized by neurodevelopmental disorders with multisystemic manifestations and distinctive facial features, presented two episodes of severe infections with sepsis. Autosomal recessive p47^{phox} deficient chronic granulomatous disease (AR47CGD) was suspected because WBS resulted from a hemizygous microdeletion at the chromosomal location 7q11.23 close to the *NCF1* gene encoding p47^{phox}, a cytosolic factor of the phagocytic NADPH oxidase complex.
Methods and results: The NADPH oxidase activity measured by NBT reduction and a fluorimetric method was abolished in the patient's neutrophils whereas this was normal in his family members. Western-blot analysis revealed the absence of p47^{phox} in the patient's neutrophils. Analysis by whole genome array CGH using an Agilent 180K oligonucleotide array and analysis by FISH using a commercial ELN Abbott probe from the 7q11.23 region revealed a deletion of approximately 1·4 Mb in 7q11.22q11.23. In addition by sequencing *NCF1*, a GT deletion at the beginning of exon 2 was found, suggesting hemizygosity of recessive AR47CGD mutation in the patient. Gene-scan methods used to determine the ratio of *NCF1* and *NCF1* pseudogenes (containing the GT deletion) revealed that his father and sister were both AR47CGD carriers.
Conclusion: We describe here an extremely rare case of AR47CGD resulting from hemizygous GT deletion in exon 2 of *NCF1* associated with WBS, a contiguous gene syndrome caused by an autosomal dominant hemizygous microdeletion

located in 7q11.23 and including in our case, the loss of the *NCF1* gene. Severe clinical syndromes appeared later than in X-linked CGD as usually found in AR47CGD, and were treated by white blood cell infusion and antibiotics. However, the patient did not suffer from hypertension which is found in about 50% of young adults with WBS. Indeed the microdeletion of WBS leads to hemizygosity at ELN encoding elastin which is thought to predispose to hypertension. Then, the absence of a functional copy of *NCF1* could be a protective factor against hypertension in our patient as previously described for hemizygosity at *NCF1* in WBS patients [Del campo et al. (2006) *Am J Hum Genet*].

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The tyrosine kinase inhibitor dasatinib inhibits the functions of human phagocytes *in vitro*

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Background: Dasatinib is a potent, dual Src/Bcr-Abl inhibitor, that is used in the therapy of imatinib-resistant chronic myeloid leukemias. While the effect of dasatinib on malignant cells is well established, little is known about its effect on non-malignant cells. Here we investigated how dasatinib influences the functions of the neutrophils which are key members of the innate immune system.
Materials and methods: Human neutrophils were isolated from peripheral blood of healthy volunteers. Cells were preincubated with different concentrations of dasatinib followed by cell activation through various routes including adhesion-mediated activation, Fc-receptor ligation, chemokines and bacterial chemoattractants or incubation with live bacteria. A number of cellular functions such as superoxide release, degranulation, adhesion, cell spreading, migration, and killing of bacteria were recorded.
Results: Dasatinib dramatically reduced the responsiveness of human neutrophils to integrin- and Fc-receptor-mediated cell activation with an IC_{50} of approximately 50 nM in both those cases. Dasatinib also reduced superoxide release triggered by the bacterial peptide fMLP but, somewhat surprisingly, promoted fMLP-induced migration in a standard Transwell system. Though higher concentrations of dasatinib moderately reduced the bacterial killing capacity of neutrophils, this effect was rather moderate at concentrations below 100 nM.
Conclusions: These data suggest that, in addition to its inhibitory effect on malignant cells, dasatinib also inhibits several functional responses of human neutrophils without significant inhibition of cell migration and bacterial killing. As the neutrophil integrins and Fc-receptors are essential components of the effector phase of autoimmune arthritis, our results raise the possibility of using dasatinib as an anti-inflammatory drug.

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Extracellular reactive oxygen species do not directly decrease levels of surface thiols on phagocytes or dampen their ability to produce proinflammatory cytokines

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Background: Phagocytes that lack the ability to generate NADPH-oxidase derived reactive oxygen species (ROS) are hyperinflammatory and respond to stimulation with elevated production of proinflammatory cytokines. This hyperinflammatory phenotype corresponds with many of the clinical symptoms associated with the rare human disease chronic granulomatous disease (CGD); in CGD phagocytes are devoid of ROS produced by the NADPH-oxidase. It thus appears that ROS can dampen inflammatory signalling, but the underlying mechanism is currently unknown. One theory states that extracellular ROS from phagocytes reduce surface thiols on neighbouring lymphocytes which suppresses cellular reactivity. The aims of this study are to evaluate if extracellular ROS (i) suppress the production of proinflammatory cytokines and (ii) directly affect the level of surface thiols on the ROS-producing phagocytes.
Materials and methods: A human promyelocytic cell-line PLB-985 (WT) and a genetically modified clone PLB-985-gp91^{phox}P415H (CGD) were differentiated to phagocyte-like cells *in vitro* then ROS production was measured using an isoluminol-amplified CL system, the relative number of cell-surface thiols was analyzed by flow cytometry and cytokine production was evaluated by ELISA.
Results: Phagocyte differentiated CGD cells failed to produce extracellular ROS yet expressed significantly higher levels of surface thiols compared to WT cells. CGD cells also produced higher levels of proinflammatory cytokines than WT cells. Thiol levels and cytokine production by WT cells were not altered by addition of scavengers of extracellular ROS. Conversely, CGD cells cultured in the presence of WT cells (i.e. extracellular ROS) did not significantly diminish surface thiol levels.
Conclusions: Extracellular ROS do not directly influence surface thiol levels on phagocytes or suppress the ability of phagocytes to produce proinflammatory cytokines.

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Src-family kinases are required for the development of autoimmune arthritis

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Background: We have previously shown that the Src-family kinases Hck, Fgr and Lyn are required for *in vitro* integrin- and Fc-receptor-mediated responses in neutrophils. Since both pathways play important roles in the development of autoimmune arthritis, we tested whether Src-family kinase-deficient mice are protected from *in vivo* experimental models of autoimmune arthritis.
Materials and methods: The K/BxN experimental mouse model of autoimmune arthritis was used to investigate the role of Src-family kinases *in vivo* using knockout mice. Arthritis was followed by clinical scoring, joint thickness measurement and a joint functional test. Bone marrow chimeras were generated having both wild-type and knockout cells in the same animal and the ratio of wild-type and knockout neutrophils migrating into the site of inflammation was compared using flow cytometry.
Results: Mice lacking all Hck, Fgr and Lyn treated with arthritogenic K/BxN serum failed to develop visible inflammation of the hind paws, joint thickening and loss of joint function due to inflammation. Hck, Fgr, Lyn single and Hck$^{-/-}$ Fgr$^{-/-}$, Fgr$^{-/-}$ Lyn$^{-/-}$ double knockouts, however, responded normally to arthritogenic treatment, although a slight defect was observed in Fgr$^{-/-}$ Lyn$^{-/-}$ double knockouts. Neutrophils

lacking all three Src-family kinases migrated normally into the arthritic site of inflammation.

Conclusions: Our results suggest that the three Src-family tyrosine kinases, Hck, Fgr and Lyn are required for the *in vivo* development of autoimmune arthritis. Neither Hck, Fgr, nor Lyn is fully responsible for the arrest of autoimmune response thus suggesting a possible compensation mechanism among various Src-family kinases *in vivo*. Src-family kinases are not required for the migration of neutrophils into the site of inflammation *in vivo*.

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M-ficolin, a pattern recognition receptor and a complement-activating humoral pattern recognition molecule

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Background: We wish to expand the knowledge on M-ficolin, which presents an enigma being both a humoral pattern recognition molecule, initiating the lectin pathway of complement, and present as a pattern recognition receptor anchored onto the G-protein-coupled receptor 43 of monocytes.

Materials and methods: Quantification of M-ficolin by TRIFMA in serum and in extracts of monocytes and granulocytes. Expression by flow cytometry. Association with infections in neonates. Quantitative analyses of binding to bacteria.

Results: M-ficolin is present in serum from blood donors at about 1 μg mL^{-1}. Flow cytometry shows presence on monocytes and to a lesser degree on neutrophiles. Extracts of monocytes contains 3×10^6 molecules of M-ficolin per cell, with about fivefold less in extracts of PMNs. The cord blood concentration is about half of that in adults. It is significantly higher in cord blood from infants with sepsis within the first 3 days after birth, and its release from leukocytes when incubated with *E. coli*. M-ficolin binds to encapsulated but not to unencapsulated *Streptococcus Agalactiea*, while the reverse was true for mannan-binding lectin.

Conclusion: The biological role of M-ficolin remains to be revealed, but despite inconsistent reports in the literature, a function in the innate immune defence appears indicated.

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A polymorphic variable number tandem repeat and human leukocyte antigen regions in gestational choriocarcinoma evaluated by polymerase chain reaction amplification

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Background: Gestational choriocarcinoma (GC) is a rare, extremely malignant type of epithelial tumour arising from the trophoblast. A complete hydatidiform mole (CHM) is the most common precursor of GC. There are two types of CHM: heterozygous and homozygous. The heterozygous CHM carry an increased predisposition to transformation to choriocarcinoma. Polymerase chain reaction (PCR) analysis of polymorphic DNA regions enables to distinguish the two forms which have great clinical importance. A variable number tandem repeat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Human leukocyte antigens (HLA) are histocompatibility antigens (glycoproteins) on the surface of nucleated cells determined by a region on chromosome 6 bearing several genetic loci. They are important in cross-matching procedures and are partially responsible for the rejection of transplanted tissues when donor and recipient HLA do not match.

Material and methods: Using PCR amplification we investigated the highly polymorphic VNTR and HLA regions in trophoblast epithelial cells from a total of 10 human biopsy tissue specimens with GC, as well as in cells from peripheral blood of patients and their partners. The PCR products were analyzed by agarose gel electrophoresis.

Results: Nine out of 10 samples were found to be of heterozygous origin, only one choriocarcinoma was of homozygous origin.

Conclusions: We have proved that both, VNTR and HLA analysis by the means of PCR amplification, are precisely reliable procedures suitable for the differential diagnosis of a CHM. Both methods enable to determine the origin of GC, however, the VNTR method is more simple to perform and less expensive.

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Kindlin-3 at the hematopoietic plasma membrane: a crucial regulator of integrin activation

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Background: Kindlin-3 is expressed in hematopoietic cells and is involved in adhesion by regulating the activation of integrins. Deficiency for kindlin-3 causes Leukocyte Adhesion Deficieny-1/variant syndrome (LAD-III), manifesting by non-pussing infections combined with a Glanzmann-like bleeding disorder and thereby demonstrating the relevance of kindlin-3-mediated adhesion for several cell types. In neutrophils, kindlin-3 is essential for uptake of pathogens, e.g. yeast particle zymosan, in a process called phagocytosis. Kindlin-3 regulates integrin activation through binding to the integrin β chain.
Materials and methods: We performed neutrophil fractionations with mild lysis buffers to separate several cell fractions. We used confocal live cell microscopy to show translocation *in vivo* and Fluorescence Recovery After Photo bleaching (FRAP) analysis to determine the speed and stability of translocation.
Results: Despite the critical role of kindlin-3 at the cell membrane, recent investigations show that the majority of kindlin-3 is localized in the cytoplasm. Neutrophil fractionations show that stimulation with pathogens induces translocation of kindlin-3 to the plasma membrane, as was confirmed using *in vivo* confocal microscopy. FRAP analysis showed that kindlin-3 at the membrane was highly motile.
Conclusions: Kindlin-3 translocates from the cytoplasm to the cell membrane during integrin activation where it is involved in a transient interaction with integrins or the plasma membrane.

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Carcinoma origin dictates functional macrophage phenotype

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Background: Macrophages are versatile cells, which phenotype is profoundly influenced by their environment. The pro-inflammatory classically activated or M1 and the anti-inflammatory alternatively activated or M2 macrophages represent the two extremes of a continuum of functional states. Consequently, macrophages that are present in tumors can exert either tumor promoting or anti-tumor activity, depending on the tumor micromilieu. Interestingly, presence of macrophages in breast or colon carcinomas correlates with poor or good prognosis in patients, respectively.
Methods and results: In this study we demonstrate that human monocytes, cultured in supernatants of colon carcinoma cells have increased reactive oxygen species (ROS) production after stimulation with LPS and IFNγ, whereas monocytes cultured in supernatant of breast carcinoma cells failed to do so. Additionally, expression of the pro-inflammatory cytokines IL6, IL12 and TNFα on both mRNA and protein levels was upregulated as well. By contrast, breast carcinoma supernatant stimulated monocytes increased expression of IL10, IL8, and the chemokine CCL22, which are associated with an alternatively activated state of macrophages.
Conclusions: Thus, colon carcinoma cells are able to predispose monocytes, polarizing them towards a more classically activated anti-tumorigenic phenotype, whereas breast carcinomas direct development of monocytes towards an alternatively activated macrophage phenotype. This discrepancy in macrophage activation by either colon or breast carcinoma cells may explain the dichotomy between patient prognosis and macrophage presence in these different tumors. Using tumor-cell secretome analysis to identify which proteins are secreted by the different colon or breast carcinoma cells several possible candidates have popped up which can explain this dichotomy in macrophage activation. Proteins found exclusively in colon carcinoma cell secretomes are known to be associated with increased macrophage activation. These proteins can in the future be used to design new therapies, directing the development of monocytes towards M1 activated tumor macrophages in cancer patients, which can have great clinical benefits.

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Non-apoptotic neutrophil cell death after phagocytosis of immunoglobulin A-opsonized bacteria

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Background: Immunoglobulin A (IgA) is the most prominent antibody in mucosal areas. Recently we demonstrated that IgA induces neutrophil migration. Therefore, IgA plays an important role in neutrophil-mediated clearance of invading pathogens. According to the current dogma, neutrophils will then go into apoptosis, and are taken up by macrophages, which initiates an anti-inflammatory program. However, this process is not beneficial at the onset of infections when micro-organisms are still present. We therefore hypothesized that neutrophils die through a different mechanism after uptake of IgA-coated bacteria.
Materials and methods: Primary human neutrophils were incubated with *E. coli* in the absence or presence of IgA. Cell death was assayed at several time points (0–24 h) with life-cell imaging using the apoptosis marker AnnexinV and cell death marker 7-AAD. Additionally, apoptosis was assessed by staining cleaved caspase 3. Morphology of cells/nuclei was examined with light and electron microscopy.
Results: Phagocytosis of *E. coli* by neutrophils was significantly enhanced in the presence of IgA. This led to rapid cell death (< 4 h), which was characterized by dispersion of nuclear contents into the cytoplasm, and 7-AAD positivity. By contrast, neutrophils that had not phagocytosed bacteria died at later time points (> 8 h), and showed hallmark apoptotic features like fragmented nuclei and AnnexinV positivity.
Conclusions: Thus, neutrophils that phagocytosed IgA-opsonized *E. coli* died through a non-apoptotic pathway. Recently, a novel way of neutrophil cell death was described that is referred to as NETosis and which is characterized by release of nuclear and cytoplasmic contents (neutrophil extracellular traps; NETs). In our experiments we observed intracellular spread of nucleic contents, and we are currently investigating whether this is also accompanied by extracellular release of NETs.

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Mechanisms of phagocytosis in macrophages: cross-talks between NFkB signaling, actin and endocytosis

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Background: Specialised cells of the immune system such as macrophages and dendritic cells are characterized by efficient internalization mechanisms, phagocytosis and macropinocytosis. Phagocytosis in macrophages is receptor-mediated and relies on actin polymerization coordinated with the focal delivery of intracellular membranes that is necessary for optimal phagocytosis of large particles. Recycling endosomes positive for VAMP3, transferrin receptor and AP1, are involved in this focal exocytosis under the control of the small GTP-binding protein ARF6. How and where vesicles are exocytosed in the phagocytic cup as compared to actin polymerization remains unclear.
Materials and methods: We set out to analyze actin remodeling and exocytosis events during pseudopods extension using Total Internal Reflection Fluorescence Microscopy (TIRFM) during phagocytosis in living macrophages. In addition, we investigated how the signaling component Bcl10, known to act in the NFkB activation pathway induced by surface phagocytic receptors, is involved in the early signaling to actin and membrane remodeling. These experiments were performed in murine macrophages, primary human monocytes and macrophages.
Results: We highlight here where and how vesicle delivery occurs in the phagosome during pseudopod extension and actin polymerization. In addition, we proved novel evidence for direct links between early events in phagosome formation and proinflammatory signaling.
Conclusion: This work reveals new molecular links in the very first steps of the innate and adaptive immune responses in monocytes/macrophages.

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Mechanisms of microbial tolerance in phagocytes

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Background: The molecular mechanisms underlying the fatal inflammatory processes of sepsis are only partly understood. Septic inflammation is triggered by conserved Pathogen Associated Molecular Patterns (PAMP), which are recognized by Pattern Recognition Receptors (PRRS) e.g. Toll-like-Receptors (TLRs). Pretreatment of phagocytes with suboptimal doses of PAMPs e.g. LPS induce a state of tolerance resulting in a second phase of hypoinflammation in sepsis. Myeloid-related Proteins 8 and 14 (MRP8/14) which are released during stress reactions by phagocytes have been described as endogenous TLR4-activators [Vogl et al. (2007)]. We investigated the molecular mechanisms responsible for the cross-talk of endogenous MRP8/14- and PAMP dependent activation in microbial tolerance.
Materials and methods: Microbial tolerance was induced by pre-treatment of phagocytes with either LPS or MRP8/14. Inflammatory response was quantified by release of cytokines. Expression levels of TLR4 were analysed by FACS and RT-PCR experiments. mRNA-levels and -stability of different cytokines were determined by RT-PCR experiments.
Results: We found that pre-activation of TLR4 by LPS or MRP8/14 induces tolerance, accompanied by decreased levels of proinflammatory cytokines. FACS and RT-PCR experiments revealed that the expression of TLR4 itself is unaffected by tolerance induction. In contrast, RT-PCR experiments showed a clear decrease of mRNA-levels of different pro-inflammatory cytokines e.g. TNFα, although the stability of the mRNA is unaffected in tolerant cells.
Conclusions: MRP8/14 and LPS induce self- and cross-tolerance via activation of the TLR4 signaling pathway. This tolerance is not caused by altered expression levels of TLR4 itself, but nonetheless results in decreased cytokine-expression and secretion. These data point towards an aberration of signaling pathways downstream of TLR4.

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Signal regulatory protein α controls phagocyte NADPH oxidase function by limiting the expression of gp91^{phox}

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Background: The phagocyte NADPH oxidase mediates oxidative microbial killing in granulocytes and macrophages. However, because the reactive oxygen species (ROS) produced by the NADPH oxidase can also be toxic to the host, controlling its activity is essential. Until now little is known about the endogenous mechanism(s) that limit NADPH oxidase activity.
Materials and methods: Myeloid cell line PLB was chosen to over express a SIRPα. On this model the production of hydrogen peroxide was measured by Amplex Red. We also used a SIRPα mutant mouse that lacks the cytoplasmic oart of the protein.
Results: Here, we demonstrate that the myeloid inhibitory receptor signal regulatory protein-α (SIRPα) acts as a negative regulator of the phagocyte NADPH oxidase. In particular, phagocytes isolated from mice lacking the SIRPα cytoplasmic tail were shown to have an enhanced respiratory burst. In line with this, the overexpression of SIRPα in human myeloid cells prevented respiratory burst activation after either granulocytic or monocytic differentiation. The inhibitory effect required interactions between SIRPα and its natural ligand CD47 as well as signaling through the SIRPα cytoplasmic ITIM motifs. Suppression of the respiratory burst by SIRPα was caused by a selective repression of gp91^{phox} expression. We are currently investigating which transcriptional factors act downstream of SIRPα and by which mechanism SIRPα regulates gp91^{phox} transcription.
Conclusions: Collectively, these results demonstrate that SIRPα can limit the expression of gp91^{phox} and thereby controls the magnitude of the respiratory burst in phagocytes.

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Increased microRNA-155 increased TNF-alpha production in Kupffer cells mediates in alcoholic liver disease

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Background: Activation of Kupffer cells by gut-derived LPS and TLR4/LPS-mediated increase in TNFα production has a central role in the pathogenesis of alcoholic liver disease (ALD). Micro-RNA (miR)-125b, miR-146a and miR-155 can regulate inflammatory responses to lipopolysaccharide (LPS). Here we evaluated the involvement of miRs in alcohol-induced macrophage activation.

Materials and methods: KCs were isolated from pair-fed or ethanol-fed mice. miRs expression was analyzed using a Taq-Man miRNA system. RAW 264·7 macrophages were used for *invitro* studies.

Results: Chronic alcohol treatment *in vitro* resulted in a time-dependent increase in miR-155 but not miR-125b or miR-146a levels in RAW264·7 macrophages. Furthermore, alcohol pretreatment augmented LPS-induced miR-155 expression in macrophages. We found a linear correlation between alcohol-induced increase in miR-155 and TNFα induction. In a mouse model of ALD, we found a significant increase in both miR-155 levels and TNFα production in isolated Kupffer cells compared to pair-fed controls. The mechanistic role of miR-155 in TNFα regulation was indicated by decreased TNFα levels in alcohol-treated macrophages after inhibition of miR-155 and by increased TNFα production after miR-155 over-expression, respectively. We found that miR-155 affected TNFα mRNA stability because miR-155 inhibition decreased while miR-155 overexpression increased TNFα mRNA half-life. Using the NF-kB inhibitors, MG-132 or Bay11-7082, we demonstrated that NF-κB activation mediated the upregulation of miR-155 by alcohol.

Conclusion: Our novel data demonstrates that chronic alcohol consumption increases miR-155 in macrophages via NF-κB and the increased miR-155 contributes to alcohol-induced elevation in TNFα production via increased mRNA stability.

Workshop 2: Poor response to gonadotrophin stimulation & primary ovarian insufficiency: a continuum?

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Follicular fluid leptin content correlates with insulinemia in normalweight women with polycystic ovary syndrome undergoing *in vitro* fertilization/embryo transfer

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Background: Polycystic ovary syndrome (PCOS) is commonly associated with anovulation and infertility. Both hyperinsulinemia/insulin-resistance and leptin-resistance seem to be involved in the pathogenesis of PCOS. We evaluated leptin levels and fertilization rate in normalweight (NW) women with PCOS (PCOS-W) undergoing *in vitro* Fertilization/embryo transfer (IVF).

Materials and methods: We recruted 16 NW PCOS-W and 7 NW normal ovulating women (Cn). All women underwent IVF according to DePalo's protocol (Gynecol Endocrinol. 2009). Estrogen and insulin levels were measured in serum and, leptin levels in both serum and follicular fluid (FF-leptin) (EASIA). For statistical analysis, Student's *t*- and Pearson tests and linear regression were applied. Significant level was $P < 0{\cdot}05$.

Results: Insulin and estrogen levels were significantly higher in PCOS-W than in Cn. In PCOS-W, leptin was significantly higher in FF ($P = 0{\cdot}009$) than in serum. In Cn, leptin was not different in FF as compared with serum. FF-leptin was not different in PCOS-W as compared with Cn, but serum leptin was lower in PCOS-W than in Cn ($P = 0{\cdot}03$). In PCOS-W, significant correlations were found between FF-leptin and both BMI ($P = 0{\cdot}022$ $R = 0{\cdot}32$) and insulinemia ($P = 0{\cdot}0058$, $R = 0{\cdot}744$), but no correlation was found between FF-leptin and estrogens. In Cn, FF-leptin was not correlated with BMI, insulinemia and estrogens. Fertilization rate was significantly lower in PCOS-W ($P = 0{\cdot}038$) than Cn, but not correlated with FF-leptin levels.

Conclusions: In NW PCOS-W, FF-leptin levels are not directly influencing fertilization rate, but insulinemia and BMI might be crucial in determining leptin gradient (serum/FF) and reducing fertilization rate.

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Predictive value of basal antral follicle count in controlled ovarian hyperstimulation for assisted reproductive technologies

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Background: This study was designed to assess the capability of antral follicle count (AFC) as a useful predictor of ovarian response and stimulation quality in assisted reproductive technologies (ART).

Materials and methods: One hundred and twelve consecutive FIVET/ICSI cycles were enrolled. Patients with endometriosis, previous ovarian surgery or polycystic ovary syndrome (PCOS) were excluded. Basal AFC by transvaginal ultrasonography and basal hormone levels were determined on the third day before the initiation of gonadotropin stimulation with a long GnRH analogue protocol. The maturity of oocytes (MII oocytes) retrieved was assessed according to Veeck. Patients were divided into four groups based on AFC, 1–5, 6–10, 11–15, > 16 antral follicles. Patients and *in vitro* fertilization (IVF) cycle performance characteristics were afterwards analyzed with respect to AFC group. Data were analyzed with the use of 'MedCalc'. Statistical significance for all tests was set at $P \leq 0{\cdot}05$.

Results: Table 1 shows patients' characteristics in relation with AFC categories while Table 2 shows cycles' characteristics by stimulation protocol in relation with AFC categories. AFC significantly correlated negatively with age, basal FSH, duration of induction and total dosage of gonadotropin and correlated positively with number of oocytes retrieved. No significant correlations were observed with Body Mass Index (BMI), basal E2 and duration of infertility.

Conclusions: AFC is a noninvasive and easy to perform method that provides predictive information on ovarian responsiveness before stimulation. If we identify women with a high risk of producing a poor response as well as those who still produce enough oocytes to have a good chance of pregnancy we can individualize the management, by stimulation dose, by counselling against initiation of IVF treatment or pertinent refusal to accept initiation.

Table 1 Patients' characteristics in relation with AFC categories

	Sub-jects (*n*)	Age (y)	BMI (kg m^{-2})	Day 3 FSH (IU mL^{-1})	Day 3 E2 (pg mL^{-1})	Duration of infertility (years)
Overall	112	34·7 ± 5	23·7 ± 3·2	6·8 ± 2·4	44·1 ± 29·8	3·4 ± 2·1
AFC 1–5	20	37·2 ± 4·8	23·3 ± 3·5	8·4 ± 2·1	49·8 ± 35·3	3·1 ± 2·6
AFC 6–10	40	35·3 ± 5	23·8 ± 3·3	6·6 ± 2·6	41·5 ± 28·5	4 ± 2·7
AFC 11–15	28	33·5 ± 4·7	23·4 ± 3·2	6·3 ± 2	43·8 ± 30·6	2·8 ± 1·8
AFC > 16	14	32·9 ± 4·2	24·8 ± 3·2	6 ± 2·3	44·2 ± 20·2	3·2 ± 1·8
P value		0·0250	0·5523	0·0069	0·7887	0·1599

Table 2 Cycles' characteristics by stimulation protocol in relation with AFC categories

	Subjects (*n*)	Duration of induction (days)	Total gonadotropin dose (IU)	MII oocytes
Overall	112	12·2 ± 1·8	2276 ± 723·3	4·7 ± 2·2
AFC 1–5	20	12·5 ± 1·3	3140 ± 713·5	3·8 ± 2
AFC 6–10	40	12·8 ± 1·6	3058 ± 691·4	4 ± 2
AFC 11–15	28	11·7 ± 1·7	2512 ± 577	5·6 ± 2
AFC > 16	14	11·3 ± 2·1	2165 ± 458·8	5·8 ± 1·4
P value		0·0076	<0·0001	0·0005

Workshop 3: Clinical investigation of obesity

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White and brown adipose tissue

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The morphology of adipose tissues is not the same throughout the body but it varies according to its location. Classically, adipose tissue is divided into two types: the white adipose tissue (WAT) and the brown adipose tissue (BAT). The difference between WAT and BAT is the vasculature (it is more dense in BAT) and the morphology and function of their adipocytes. The adipocytes in WAT contain a single large depot of stored triglycerides (unilocular) while the adipocytes in WAT have a fragmented depot of triglycerides (multilocular). Characteristically, the adipocytes in BAT are extremely rich in mitochondria which contain large amounts of the uncoupling protein-1 (UCP-1) which is located within the inner mitochondrial membrane and allows protons to escape mitochondria thus preventing them to produce high energy compounds like ATP and instead loose it as heat.

In general, the role of WAT adipocytes is to store energy (in the form of triglycerides) while the role of BAT adipocytes is to spend energy (in the form of heat). In addition to their handling of energy, white adipocytes differ from brown adipocytes is several other aspects including expression of several developmental genes, response to insulin, response to adrenergic innervation, density of sympathetic innervation, production of immune system effectors and production of hormones. For example, leptin is mainly produced by BAT while WAT produces mainly adiponectin, tumor necrosis alpha (TNFa), interleukin 1 (IL1), IL6, IL-8, visfatin, resistin, omentin, the acute phase protein serum amyloid A and plasminogen activator inhibitor (PAI-1). In addition, the size of adipocytes in the WAT also affects their expression of hormones and inflammatory mediators including the production of IL6, IL8, granulocyte colony stimulating factor (G-CSF) and the chemokine monocyte attractant protein-1 (MCP-1) or C-C motif chemokine ligand-2 (CCL2).

In a normal adult human WAT is localized subcutaneously, in the pericardium, within the muscles (intramuscularly), and within the abdomen in the omental, retroperitoneal and visceral fat depots. On the other side, BAT is detectable in the cervical, supraclavicular, ax>illary, and paravertebral regions by positron emission tomography (PET) scans of metabolically active fat. However, it now appears that adipocytes within the WAT can be induced to change into a brown phenotype. Indeed, treatment with thiazolidinediones, beta adrenergic agonists, or Undaria pinnatifida (a seaweed) can induce WAT adipocytes to express more UC-1 protein and less pro-inflammatory genes. Similar outcomes have been documented following loss of weight, acute or chronic exercise or exposure to cold or sunlight.

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Models of obesity: genetics and beyond.

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Obesity is a progressive world-wide epidemic reaching alarming rates and is strongly associated to parallel rise in type 2 diabetes. One of the main questions in the field is why expansion of white adipose tissue is associated with increased susceptibility to different diseases and how this susceptibility is determined in different individuals. We will present the theories and hypotheses on the above questions and review supporting data from experimental studies in model organisms and from clinical studies. We will also present experimental models of obesity in an effort to elucidate its pathophysiology. Finally, we will discuss data from human genetic studies and thoughts on the usefulness of systems medicine applications in the prevention, diagnosis and treatment of this condition in humans.

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Serum adiponectin and resistin improve only after gastric banding plus lifestyle changes

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Background: Adiponectin and resistin are adipokines involved in the chronic low-grade inflammation associated with

Obesity. In humans, adiponectin and resistin circulating levels positively correlate with insulin sensitivity and cardiovascular risk respectively.

Materials and methods: In 27 severely obese non-diabetic insulin-resistant subjects (SevOb) (BMI ≥ 35 Kg m^{-2}), waist, total cholesterol (T-Col), HDL, LDL, triglycerides, serum adiponectin and resistin (Elisa), % excess weight loss (%EWL), $HOMA_{IR}$ were evaluated before (T0) and 6 (T6), and 12 (T12) months after laparoscopic gastric banding (LapGB) plus an education program to promote lifestyle changes. Serum adiponectin and resistin were also measured in nine healthy controls.

Results: At T0, resistin levels were significantly higher ($P = 0{\cdot}006$) and adiponectin levels significantly lower ($P = 0{\cdot}0009$) in SevOb than controls. In SevOb, BMI, waist, T-Col, LDL, triglycerides, $HOMA_{IR}$ were significanly lower and HDL significantly higher at T6 and T12 than at T0. At T12, 14 SevOb out of 27 (group 1) showed %EWL ≥ 40%, significantly increased adiponectin levels ($P = 0{\cdot}0032$) and significantly decreased resistin levels ($P < 0{\cdot}0038$) as compared with T0. The remaining 13 subjects (group 2) showed %EWL < 30%, significantly decreased adiponectin levels ($P = 0{\cdot}027$) and no changes in resistin as compared with T0. At T12 SevOb of group 1 showed adiponectin levels still significantly lower than controls ($P = 0{\cdot}002$) but resistin levels not significantly different from those of controls. SevOb of group 1 showed a perfect compliance to lifestyle changes, those of group 2 a poor compliance.

Conclusions: Only LapGB-induced %EWL ≥ 40% plus lifestyle changes is associated with a significant improvement in adiponectin and resistin circulating levels in SevOb.

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Health status investigation in elementary school students at Crete: Anthropometry, fitness, biochemical markers

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Background: Children constitute the population group which should be given particular attention, so that 'obesity epidemic' will be controlled in the future.

Aim: The recognition of the risk factors for cardiovascular diseases, the evaluation of physical fitness and mental health of examined population as well as comparison with corresponding studies that have been carried out in Greece and abroad.

Method: Ninety pupils, 11 years old, were examined; studying in seven different schools of Siteia. Data were obtained on pupil's anthropometry (weight, height, Body Mass Index, waist circumference and hip circumference); three days recall of physical activity (2 working days and one holiday) and blood samplings. The children participated in physical fitness tests (endurance shuttle run test, handgrip power test, and falls back from supine sprawl) and completed two tests for mental balance evaluation. The statistical analysis of data was carried out with the help of program 'SPSS 13.0'.

Results: Totally in the children that participated in the study, 18·9% were obese (BMI greater than the 95th percentile) and 17·8% were overweight (BMI > 85th percentile). Concerning fitness, 76·5% of children that did not participate in organised physical activity were obese. Also it appeared that the children with high rate of television viewing and computer use did not participate in organised physical activity. Finally, obesity correlates negatively with mental health balance. With regard to the biochemical markers, total cholesterol, levels of glucose and atherogenic index (LDL - C/HDL - C) were positively correlated to BMI values.

Conclusions: Because of the recognized negative consequences of obesity and decreased levels of physical activity, application of a prevention and intervention program from early school age is essential.

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A short aerobic training program reduced Tumor Necrosis Factor-alpha in obese adults with Down syndrome

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Background: The presence of the so-called 'low-grade' inflammatory state is recognized as a critical event in adipose tissue dysfunction in obesity. Recent studies have reported aerobic training may reduce proinflammatory status in patients with metabolic syndrome and type-II diabetes. This finding has been explained, at least in part, since aerobic training reduced fat mass. However to our knowledge there is no information regarding its influence in cytokine levels in individuals with Down syndrome. Accordingly the present study was undertaken to ascertain the influence of aerobic training in plasmatic Tumor Necrosis Factor-alpha (TNF-α) in young adults with Down syndrome.

Materials and methods: Thirty young adults with Down syndrome (23·6 ± 1·8 years-old;167·1 ± 5·2 cm;83·9 ± 4·6 kg) performed a 12-week training program in treadmill, 3 days per week, consisting of warm-up (15 min), main part (20–35 min) at a work intensity of 60–75% of peak heart rate [HRmax = 194 ·5-(0·56 × age)] and cool-down (10 min). Control group included six age, sex and BMI-matched individuals with trisomy 21 that did not perform any training program. Plasmatic Tumor Necrosis Factor-alpha (TNF-α), was assessed by ELISA (Immunotech, Coulter Corp., Westbrook, MA, USA). Fat mass percentage was determined by bioelectric impedance method. This study complied with the ACSM statement regarding the use of human subjects and informed consent.

Results: When compared to baseline TNF-α was decreased significantly after our 12-week protocol (9·5 ± 1·8 pg mL^{-1} vs. 7·1 ± 1·5 pg mL^{-1}; $P < 0{\cdot}001$). Similarly fat mass percentage was also reduced in experimental group (33·6 ± 3·2% vs. 31·9 ± 3·0%; $P < 0{\cdot}05$). We also found a significant association between both parameters ($r = 0{\cdot}52$; $P < 0{\cdot}05$) Conversely, no changes were reported in controls (9·3 ± 1·7 pg mL^{-1} vs. 9·1·0 ± 1·8 pg mL^{-1}; $P > 0{\cdot}05$).

Conclusion: A 12-week aerobic training program decreased significantly plasmatic TNF-α, in adults with Down syndrome. Future studies are required.

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Progressive resistance training increased glutathione peroxidase activity in an obese type-II diabetic rat model

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Background: The present study was designed to assess the influence of a 12-week progressive resistance training on

glutathione peroxidase activity in gastrocnemius muscle of obese type-II diabetic rats.
Materials and methods: Twenty-eight male spontaneously hypertensive/NIH-corpulent rats (SHR/NDmcr-cp) aged 5-weeks, were divided into two groups as exercised (n = 18) and controls (n = 10). Rats were housed in individual cages in a temperature- and light-controlled environment, having free access to high carbohydrate/low fat diet with tap water containing 30% sucrose). Animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals based upon the Helsinki Declaration. Resistance exercise was performed using a weight-lifting exercise model. Rats fitted with a canvas jacket were able to regulate the twisting and flexing of their torsos and were suspended in a standard position on their hindlimbs. They flexed their legs repeatedly, which lifted the weight-arm of the training apparatus. Training started after 2-weeks of adaptation and after measurement of the maximum weight lifted (1-repetition-maximum) with the squat-training apparatus. Training load was set at 65% of 1-repetition-maximum (RM) and it was raised 5% each 4 weeks. Rats were exercised with 3-sets of 10-repetitions, each with a 120-s rest period between sets, three times per week for 12 weeks. Tissue samples (Gastrocnemius muscle) were thawed in 50-mM ice-cold phosphate buffer and homogenized at 0 °C using supernatants for analysis. Glutathione peroxidase activity (GPx; EC1.11.1.9) was assessed spectrophotometrically.
Results: Compared to baseline GPx activity was increased significantly after resistance training (30·1 ± 2·1 U g^{-1} vs. 37·7 ± 2·3 U g^{-1} protein; $P < 0·001$). No changes were reported in controls (29·8 ± 1·9 U g^{-1} vs. 30·3 ± 1·8 U g^{-1} protein; $P > 0·05$).
Conclusion: Progressive resistance training increased GPx activity in muscle rats. Further studies are required.

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The influence of 30 days injection of monosodium glutamate on body weight and structural-functional state of gastric mucosa in rats

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Background: Well known flavor enhancer – monosodium glutamate (MSG), E 621 is widely used in food industries. However, its excessive consumption causes of 'Chinese Restaurant Syndrome'. We chose doses 15 and 30 mg kg^{-1} (correspond to 1 and 2 g for human) because due to the literature 1 g MSG has no inauspicious action on the human, but 3 g are hazardous to health. The aim: to study the effects of 30 days injection of MSG on body weight, gastric acid secretion (GAS), gastric mucosa (GM) in rats.
Materials and methods: The study was carried out on 63 rats, which were divided into three groups. I – control (during 30 days injected 0·5 mL water per os). The rats of II and III group during 30 days received 15 and 30 mg kg^{-1} of MSG, consequently.
Results: It was established that 30-day treatment with MSG in dose of 15 mg kg^{-1} leads to increase of body weight by 100% ($P < 0·001$) and basal GAS by 183% ($P < 0·05$). In GM of these rats ulcers (area – 4·0 ± 0·22 mm^2) and erosions (length – was 2·57 ± 0·2 mm) were developed. Increase of dayly dose of MSG to 30 mg kg^{-1} enhanced the damages of GM, body weight by 153% ($P < 0·001$) and GAS by 372% ($P < 0·05$).
Conclusions: (i) Long-term consumption of MSG leads to obesity, hypersecretion, erosive and ulcerative lesions in GM; and (ii) Hypersecretion of hydrochloric acid stimulate appetite and may be one of mechanism of obesity development.

Workshop 4: Innate immune system in health and disease

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Emerging role of myeloid-derived suppressor cells in the regulation of autoimmune diseases

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Autoimmune diseases are often characterized clinically by periods of exacerbation followed by remission. Immune regulatory cells are critical contributors to peripheral tolerance however mechanisms underlying the natural suppression of inflammation remain elusive and could be exploited for therapeutic purposes. Myeloid-derived suppressor cells (MDSCs) comprise a heterogenous population of myeloid precursors of macrophages, dendritic cells and granulocytes that are characterized by the co-expression of the Gr-1 antigen and the α_M integrin CD11b. Extensive studies have established a prominent role of MDSCs in the regulation of adaptive immune responses in mice during cancer, infections and transplantation whereas in humans, MDSC accumulation at tumor site, down-regulate antitumor immunity promoting therefore tumor surveillance and growth. Although the role of MDSCs in tumor immunology is well characterized, their role in the regulation of autoimmune responses is less well defined, as myeloid precursor cells with the MDSCs phenotype have been shown to play either pathogenic or regulatory role. Most importantly, the contribution of MDSCs in human autoimmune diseases remains unclear. Delineating the role of MDSCs in the regulation of autoimmune diseases opens new avenues for the development of more cell-specific based therapies in patients with autoimmune inflammatory diseases.

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The role of micro RNAs and Akt kinases in innate immune responses

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MiRNA expression profile of macrophages is altered during macrophage activation contributing to the regulation of the inflammatory response. Recent evidence has demonstrated that miRNAs control genes that participate both in innate and adaptive immune responses. In macrophages miRNAs play a central role in regulating the response to TLR signals. MiRNAs can act as negative or positive modulators during macrophage activation controlling the expression a variety of inflammation-related genes. Work from our group has shown that miR-155 is induced upon macrophage activation and acts as a positive regulator of inflammation by targeting the mRNA of SOCS1, a protein that suppresses TLR and cytokine signaling. In contrast, let-7e is also induced upon macrophage activation and acts as a negative regulator by suppressing TLR4 expression. By modulating the expression of pro- and anti-inflammatory genes miRNAs play a central role in the development of endotoxin tolerance. The signals that mediate miRNA expression are not well known. Experiments utilizing knockout animals have shown that the Akt1 isoform of Akt kinases, a family of kinases induced upon macrophage activation, regulates miRNA expression suppressing the responsiveness of macrophages to TLR signals. In contrast, the Akt2 isoform acts as a positive regulator of macrophage activation, since its genetic ablation suppresses their responsiveness to pro-inflammatory insults and promotes their polarization to Alternatively Activated Macrophages (AAMs).

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The innate immune response in acute lung injury

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Acute lung injury (ALI) is the most common cause of respiratory failure in patients in the ICU, and a major cause of morbidity and mortality. ALI is characterized by widespread alveolar capillary leakage and neutrophil infiltration in the airspaces, resulting in respiratory failure. ALI occurs as part of a systemic inflammatory response, initiated by either infectious or noninfectious inflammatory stimuli. Activation of the innate immune system plays a central role in the development of ALI. The innate immune response is activated through families of pattern recognition receptors (PRRs). PRRs include the Toll-like receptors (TLRs), the cytosolic NOD-like receptors (NLRs), the RIG-I–like receptors (RLRs), and DNA sensors. Activation of extra- and intracellular PRRs expressed in alveolar macrophages, epithelial, and endothelial cells stimulates the production of antimicrobial peptides as well as inflammatory mediators. Several common, non-infectious clinical conditions such as trauma, ischemia, aspiration, high-stretch mechanical ventilation, activate the immune response and promote ALI by releasing endogenous damage-associated molecular patterns (DAMPs) recognized by the same PRRs.

There is increasing evidence for an important role of alveolar macrophages in ALI. Alveolar macrophages are the principal resident immune cells found in the air spaces. Activation of PRRs results in 'classical' activation of alveolar macrophages, an M1 response, with release of early-response cytokines and reactive oxygen and nitrogen species, which stimulates alveolar cells to produce chemokines, and recruit neutrophils and lymphocytes. The capillary leak induced by the reactive oxygen and nitrogen species, and the neutrophil infiltration in the lung give the complete clinical picture of ALI. Indeed, depletion of alveolar macrophages has been shown to reduce experimental ALI. On the other hand, alveolar macrophages have been shown to contribute to the resolution of inflammation by phagocytosis of apoptotic neutrophils and resident cells, suggesting an important role for alternatively activated, M2 macrophages in ALI.

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IL-23 producing dendritic cells (DCs) regulate fungal pathogenicity via the induction of T_H-17 responses

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The newly identified IL-23/T_H-17 axis regulates epithelial host defense against a growing list of fungi. Whether different airborne fungi trigger a common signaling pathway for T_H-17 induction, and whether this ability is related to the inherent pathogenic behavior of each fungus in currently unknown. We found that, as opposite to pathogenic fungi (*Histoplasma*), airborne opportunistic fungi (*Aspergillus* and *Rhizopus*) trigger a common innate sensing pathway in human DCs that results in robust production of IL-23 (IL-23-DCs) and drives T_H-17 responses. This response requires activation of *dectin-1* by β-glucan that is selectively exposed during the invasive growth of opportunistic fungi. Notably, unmasking of *β*-glucan in a mutant of *Histoplasma*, not only abrogates the pathogenicity of this fungus, but also triggers the induction of IL23-DCs. Thus, sensing of fungal cell wall *β*-glucan is essential for the induction of IL-23/T_H-17 axis and may represent a key factor that regulates protective immunity to opportunistic but not pathogenic fungi.

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Novel virus-host interactions

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The success of viruses as pathogens of humans depends on their ability to reprogram the host cell metabolism to support the viral infection cycle and to suppress host defence mechanisms. Apart from the well established immune response pathways, a large number of host factors have been identified and the great variety of cellular functions performed by these factors indicates the existence of a truly complex interaction between viruses and the host cell. Nuclear domains 10 (ND10), alternatively termed PML nuclear bodies (PML-NBs) have been discovered as a nuclear substructure that is targeted by a variety of viruses belonging to different viral families and accumulating evidence argues for an involvement of ND10 in host antiviral defences either via mediating an intrinsic immune response against specific viruses or via acting as a component of the cellular interferon pathway. The non-coding RNAs designated microRNAs provide a unique level of post-transcriptional gene regulation that modulates a range of fundamental cellular processes. Several viruses, especially herpesviruses, also encode miRNAs, and over 200 viral miRNAs have now been identified. Current evidence indicates that viruses use these miRNAs to manipulate both cellular and

viral gene expression. Furthermore, viral infection can exert a profound impact on the cellular miRNA expression profile, and several RNA viruses have been reported to interact directly with cellular miRNAs and/or to use these miRNAs to augment their replication potential. The role of chromatin and epigenetic regulation of virus is an emerging field in Virology since accumulating evidence reveals that viral genomes are targeted by epigenetic regulatory mechanisms (DNA methylation, histone modifications, binding of regulatory proteins) in infected cells. In parallel, proteins encoded by viral genomes may affect the activity of a set of cellular promoters by interacting with the very same epigenetic regulatory machinery. This may result in epigenetic dysregulation and subsequent cellular dysfunctions that may manifest in or contribute to the development of pathological conditions. Additionally, viral pathogens whose complex replication cycles include coupled stages of lytic replication and latency/persistence are impacted by epigenetic regulation that plays a controlling role in determining the viral state. Elucidation of the virus-host interactions at the molecular and cellular level may have important therapeutic implications leading to novel specific or broad-range resistance and antiviral tools.

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Bacterial infections, DNA virus infections, and RNA virus infections manifest differently in neutrophil receptor expression

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Background: Treating viral illnesses or non-infective causes of inflammation with antibiotics is ineffective, and contributes to the development of antibiotic resistance, toxicity and allergy leading to increasing medical costs.
Materials and methods: In this prospective study, standard clinical laboratory data (neutrophil count, serum C reactive protein level, erythrocyte sedimentation rate) and quantitative flow cytometric analysis of neutrophil complement receptors, CR1 and CR3, as well as FcγRI (CD64) were obtained from 292 hospitalized febrile patients. After microbiological confirmation or clinical diagnosis, 135 patients were found to have either bacterial (n = 89) or viral (n = 46) infection. The patient data was compared to 60 healthy controls.
Results: We described a novel marker of local and systemic bacterial infections designated 'clinical infection score (CIS) point', which incorporates quantitative analysis of complement receptors on neutrophils and standard clinical laboratory data. CIS point varied between 0 and 8, and displayed 98% sensitivity and 97% specificity in distinguishing between bacterial and viral infections. We noticed that in dsDNA virus infections (n = 21) the average amount of CD64 on neutrophils was over five-fold compared to ssRNA virus infections (n = 22). DNA virus score (DNAVS) point, which incorporates quantitative analysis of CD64 on neutrophils and total and differential count of leukocytes, varied between 0 and 8, and displayed 95% sensitivity and 100% specificity in distinguishing between dsDNA and ssRNA virus infections.
Conclusions: These findings suggest that the proposed CIS-based and DNAVS-based diagnostic tests could potentially assist physicians in deciding whether antibiotic treatment is necessary and which antiviral treatment must be chosen.

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Peptidoglycan from staphylococcus aureus Wood 46 stimulate of maturation of dendritic cell generated *in vitro*

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Background: Dendritic cells (DC) work as a natural adjuvant to elicit T-cell immunity. Though, DCs have been widely used in immunotherapy in patients with cancer. The aim of the investigation was to estimate the effect of peptidoglycan (PG) from Staphylococcus aureus Wood 46 on phenotypic and functional properties of dendritic cell generated *in vitro*.
Materials and methods: Standart protocol for the generation of human DC from monocytes of peripheral blood was used. Lymphocyte activation was evaluated in the mixed lymphocyte culture. Expression of costimulatory molecules was investigated by flow cytofluorimetry.
Results: Treatment of DC, generated from peripheral blood monocytes, with PG in concentration of 2 mg mL^{-1} resulted in increase of expression of costimulatory molecules (CD80, CD86) and essential for antigen presentation HLA-DR molecules. Mature DC stimulated proliferation allogenic T-lymphocytes in mixed culture of lymphocytes. Prolipherative index of T-lymphocytes incubated with allogenic DC, treated with PG was 48·1% (prolipherative index in probes with polyclonal activator of T-lymphocytes – phytohemagglutinin – was 42·29%).
Conclusions: PG from Staphylococcus aureus Wood 46 in the concentration of 2 mg mL^{-1} stimulates phenotypic and functional maturation of DC *in vitro*. These results suggest that PG can be used as an alternate stimulator of maturation of DC generated *in vitro*.

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The influence of multiprobiotic 'Symbiter acidophilic concentrated' on stress-induced alteration of IL-1β level in plasma of rats

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Background: Stress action causes the increase of interleukin 1β (IL-1β) level in blood that leads to rise of corticosteroids. Corticosteroids affect damages in gastric mucosa. It was shown that multiprobiotic 'Symbiter acidophilic concentrated' can accelerate healing of stress-induced injuries, but the exact mechanisms of such action are poorly understood. Therefore the aim of our work was to investigate the stress influence on IL-1β level in blood serum in different terms after stress and its possible correction by Symbiter.
Materials and methods: The study was carried out on 100 white rats. They were divided into 10 groups in 10 rats each. The animals of the first group were intact control. The rats of groups II-X were exposed to water immersion restraint stress (WIRS) by Takagi *et al*. (1964). Blood samples for measuring IL-1β level were collected from rats of control, immediately after WIRS (group II) and in 4 consequent days after WIRS (groups III-X). The animals of these eight groups were injected with 0·5 mL of water *(per os)* or 0·5 mL of water solution of Symbiter at dose 140 mg kg^{-1} twice a day during all 4 days. The level of IL-1β in blood serum we evaluated by method of enzymoimmunoassay.

Results: It was investigated that Symbiter significantly decreased IL-1β level in blood serum of rats which increased in conditions of stress action. This effect was evident on the second day: IL-1β level was lower by 27% ($P < 0{\cdot}05$) in comparison with group that got water. In 3 days IL-1β level in blood serum of rats which got probiotics didn't differ from that in control group while in groups that got water this rate was higher by 62% ($P < 0{\cdot}01$) and by 50% ($P < 0{\cdot}05$) on third and fourth days in comparison with control group.
Conclusions: Injection of multiprobiotic Symbiter to rats after stress action decreseses the increased level of IL-1β in the blood serum that may be one of the mechanisms of protective action of Symbiter on stress-evoked injuries in gastric mucosa.

Workshop 5: Steroid receptors

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Circadian rhythm transcription factor CLOCK-mediated regulation of glucocorticoid action at local target tissues: physiologic and pathophysiologic implications

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Background: Glucocorticoids, the end-products of the hypothalamic-pituitary-adrenal (HPA) axis, influence functions of all organs and tissues through the glucocorticoid receptor (GR). Their circulating levels fluctuate diurnally under the control of the 'master' circadian CLOCK located in brain hypothalamus, while influence of its peripheral component on the local glucocorticoid actions has not been known.
Materials and methods: We employed reporter assays and mRNA quantification for GR-responsive genes, protein-protein-interaction assays, chromatin immunoprecipitation and Western blots for GR acetylation. We examined GR acetylation and mRNA expression of CLOCK-related and glucocorticoid-responsive genes in peripheral blood mononuclear cells of 10 healthy subjects sampled at 8 AM and 8 PM in the same day.
Results: Clock/Bmal1 repressed GR-induced transcriptional activity, and transactivational activity of GR on an endogenous glucocorticoid-responsive gene fluctuated in a circadian fashion, mirroring with Clock/Bmal1 mRNA expression. Clock interacted with GR physically and suppressed binding of GR to glucocorticoid response elements by acetylating multiple lysine residues located in its hinge region. In human subjects, GR acetylation was higher in the morning than in the evening, synchronizing the fluctuations of Clock/Bmal1 mRNAs and circulating ACTH and cortisol. The mRNA expression of some, but not all, glucocorticoid-responsive genes fluctuated diurnally, suggest gene specificity in responsiveness to the acetylated GR.
Conclusions: Clock/Bmal1 is a reverse phase negative regulator of glucocorticoid action in target tissues, antagonizing diurnally fluctuating circulating glucocorticoids and providing a local counter regulatory loop in a gene-specific fashion. Coordinated regulation of glucocorticoid action at target tissues by circulating cortisol and peripheral CLOCK-mediated GR modulation appears to be essential for the maintenance of proper glucocorticoid homeostasis; conversely, uncoupling of these activities may lead to complex diseases through 'functional' glucocorticoid excess.

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Generalized glucocorticoid resistance and hypersensitivity states

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Background: Glucocorticoids regulate a broad spectrum of physiologic functions essential for life and play an important role in basal and stress-related homeostasis. The actions of glucocorticoids are mediated by their ubiquitously expressed glucocorticoid receptors.
Methods: The molecular basis of Primary Generalized Glucocorticoid Resistance and Hypersensitivity has been ascribed to mutations and/or polymorphisms in the human glucocorticoid receptor (hGR) gene, which impair glucocorticoid signal transduction and alter tissue sensitivity to glucocorticoids. We have identified most hGR mutations associated with Primary Generalized Glucocorticoid Resistance, and one mutation associated with Primary Generalized Glucocorticoid Hypersensitivity. We have systematically investigated the molecular mechanisms through which these various natural hGR mutants affect glucocorticoid signal transduction.
Results: All mutations affected the ability of the receptor to transactivate the glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter in response to dexamethasone. Mutations within the ligand-binding domain (LBD) of the receptor impaired mostly the affinity of the receptor for the ligand and the interaction of the receptor with the glucocorticoid-responsive interacting protein-1 (GRIP1) coactivator, while mutations within the DNA-binding domain (DBD) impaired the ability of the receptor to bind to DNA. Mutations within the LBD and DBD also resulted in delayed nuclear translocation of the receptor following exposure to the ligand.
Conclusions: Mutations in the hGR gene impair the molecular mechanisms of glucocorticoid action, thereby altering tissue sensitivity to glucocorticoids. The study of the functional defects of natural hGR mutants sheds light to the mechanisms of hGR action, and highlights the importance of integrated cellular and molecular signalling mechanisms for maintaining homeostasis and preserving normal physiology.

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Non-linear association between androgen receptor CAG-repeat length and risk of male subfertility – a meta-analysis

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Introduction: The androgen receptor (AR) contains a polymorphic CAG segment, spanning from 10 to 30 repeats that is involved in regulation of the ARs' activity. The CAG stretch has been assumed to be inversely associated with AR function, so that the activity has been thought to diminish with increasing CAG number. However, analyses of human data based on this theory have shown numerous conflicting results regarding the link between CAG number and phenotypic characteristics such as male infertility. A recent meta-analysis showed < 1 repeat longer CAG stretch in the infertile men compared to fertile counterparts when mean CAG lengths were compared.

We have recently shown that the receptor with median CAG length (CAG22) was the most efficient in driving a reporter gene compared to those in the outer normal ranges, CAG16 and CAG28. This finding generated the hypothesis that men with long or short repeats have lower androgenicity and therefore both outlier groups could have an increased infertility risk.

Materials and methods: Data from the mentioned meta-analysis was collected and re-analysed in a stratified manner. The cohort was comprising $n = 1831$ fertile and $n = 2084$ infertile men, divided into the following categories: CAG<22, CAG 22–23 (reference) and CAG>23.

Results: The stratified analysis showed that men with longer or shorter CAG repeats than the reference had approximately 20% increased risk of infertility (< 22 OR 1·18, $P = 0{\cdot}031$; > 23 OR 1·22, $P = 0{\cdot}023$).

Conclusion: The length corresponding to the median in Caucasians gave optimal AR activity, which may have a protective effect against infertility.

Workshop 6: The HDL hypothesis: From epidemiology to bench

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Lipids or apolipoproteins in predicting events; which choice?

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Estimating the risk for a first or consecutive vascular event or death from vascular causes in individual patients is of major importance for patients and doctors in clinical practice, as treatment decisions depend on anticipated absolute risk. Various risk scores and prediction algorithms exist for various patient groups. Nevertheless, reliable prediction of vascular risk is difficult and still not very accurate. Plasma lipids are important risk factors and treatment of lipids reduces vascular risk, there is a wide distribution of plasma lipid concentrations in the population and measurement of plasma lipids is generally well standardized. Therefore plasma lipids could be very useful in estimating vascular risk in individual patients. In current prediction rules total cholesterol or total cholesterol/HDL ratio are incorporated in existing risk scores (Framingham, SCORE). It could be argued that apolipoproteins are more closely related to the etiology of atherotrombosis and may therefore predict risk more accurately. Whether this is the case and how lipids can be used in prediction rules for various patient populations is topic of this presentation, as well as an overview of current methodology to develop prediction rules in clinical practice.

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HDL cholesterol, apolipoprotein A-I and high molecular weight adiponectin are decreased by dietary sodium restriction in healthy men: relationships with renal hemodynamics and RAAS activation

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Background: We determined the effect of short-term dietary sodium restriction on plasma total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides, apolipoprotein (apo) A-I, apo B and high molecular weight (HMW) adiponectin in non-obese, normotensive young men. Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), plasma renin activity (PRA) and aldosterone were also measured.

Materials and methods: Sixty-five men, aged 23 ± 7 years, were randomly studied on a high sodium intake (HS, 228 ± 77 mmol Na+ per 24 h) and a low sodium intake (LS, 36 ± 27 mmol Na+ per 24 h), each period lasting 1 week.

Results: LS decreased GFR and ERPF and increased PRA and aldosterone ($P < 0{\cdot}0001$ for all). LS also induced a decrease in HDL-C (3·8 ± 0·8%), apo A-I (3·7 ± 6·5%) and HMW-adiponectin (13·6 ± 40·5%; $P < 0{\cdot}05$ for all), but plasma total cholesterol, LDL-C, triglycerides and apo B did not significantly change. The changes in HDL-C and apo A-I were correlated negatively to the changes in ERPF ($r = -0{\cdot}25$ and $r = -0{\cdot}26$, $P < 0{\cdot}05$), whereas the changes in HMW adiponectin were correlated

negatively to the changes in PRA and aldosterone ($r = -0{\cdot}24$ and $r = -0{\cdot}27$, $P < 0{\cdot}05$).

Conclusions: Short term sodium restriction modestly decreases HDL-C and apo A-I in healthy men. This HDL-C lowering could modify the beneficial effects on cardiovascular risk consequent to sodium restriction. The inverse relation of the drop in HDL-C and apo A-I with ERPF changes suggests that functional renal hemodynamic adaptation to sodium restriction affects HDL metabolism. Stimulation of the renin-angiotensin-aldosterone system by low sodium diet is likely to decrease HMW adiponectin.

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Dyslipidemia is extremely prevalent and is the most common metabolic syndrome component among Saudi children and adults

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Background: Decreased levels of circulating high density lipoprotein cholesterol (HDL-C) is a significant risk factor for cardiovascular events and a major component of the metabolic syndrome (MetS). This study aims to determine which among the components of MetS is most prevalent in the Saudi population.

Materials and methods: A total of 4081 Saudi subjects (10–55 years old) were included. Anthropometrics were measured and fasting blood samples were collected. Fasting blood glucose and lipid profile were analyzed using routine laboratory methods. The definition of Adult Treatment Panel III (NHANES III) and its modified version were used to screen for MetS in both children ($N = 1231$) and adults ($N = 2850$).

Results: Over-all prevalence of complete MetS in children was 9·4% [Confidence Interval (CI) 7·8–11·0] and in adults 35·3% (CI 33·5–37·01). In both cohorts, low HDL-C was the most prevalent MetS component, affecting 86% (CI 85·0–88·6) of children and 88·6% (CI 87·5–89·7) of adults. Hypertriglyceridemia was the second most prevalent MetS component affecting 33% (CI 30·6–35·8)% of children and 34% of adults (CI 32·3–35·7)

Conclusions: Dyslipidemia is extremely common in both children and adult Saudis. Further investigation at the genomic, proteomic and lipidomic levels is warranted to determine the etiology and design novel target therapies.

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Relationship of plasma apolipoprotein M with proprotein convertase subtilisin-kexin type 9 levels: modification by adiposity in non-diabetic subjects

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Backgroud: Apolipoprotein M (apoM) retards atherosclerosis development in murine models, and may be regulated by pathways involved in LDL metabolism. Proprotein convertase subtilisin-kexin type 9 (PCSK9) plays a key role in LDL receptor processing. We determined whether plasma apoM is related to PCSK9 levels in subjects with varying degrees of obesity.

Materials and methods: We sought correlations between plasma apoM and PCSK9, measured using recently developed ELISAs, in 79 non-diabetic subjects [42 subjects with body mass index (BMI) ≥25 kg m^{-2}].

Results: ApoM and PCSK9 levels were both correlated positively with total cholesterol, non-HDL cholesterol, LDL cholesterol and apoB ($P < 0{\cdot}05$ to $P < 0{\cdot}001$). ApoM correlated positively with PCSK9 in lean subjects ($r = 0{\cdot}337$, $P = 0{\cdot}041$; ß = 0·413, $P = 0{\cdot}021$ after BMI and apoB adjustment), but not in subjects with BMI ≥ 25 kg m^{-2} ($r = 0{\cdot}099$, $P = 0{\cdot}53$).

Conclusions: The PCSK9 pathway may contribute to plasma apoM regulation in humans. The influence of PCSK9 on circulating apoM appears to be modified by adiposity.

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Plasma proprotein convertase subtilisin-kexin type 9 does not change during 24 h insulin infusion in healthy subjects and type 2 diabetic patients

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Background: Proprotein convertase subtilisin-kexin type 9 (PCSK9) promotes low density lipoprotein (LDL) receptor degradation, thereby providing a key pathway for LDL metabolism. PCSK9 mRNA expression may be upregulated by insulin in murine models. Here we examined effects of exogenous hyperinsulinemia on plasma PCSK9 levels in humans without and with type 2 diabetes mellitus.

Materials and methods: A 24 h moderately hyperinsulinemic glucose clamp (30 mU kg^{-1} per hour) was performed in eight healthy men and eight male type 2 diabetic patients. Plasma PCSK9 was measured using a recently developed sandwich enzyme-linked immunosorbent assay.

Results: Plasma LDL cholesterol and apolipoprotein B levels were lowered by insulin in healthy subjects and diabetic patients ($P < 0{\cdot}01$ for all), whereas triglycerides were also decreased in healthy subjects ($P < 0{\cdot}01$). Plasma PCSK9 levels remained unchanged in healthy subjects (median (interquartile range) change, −23 (−63 to 25) %, $P = 0{\cdot}50$) and in diabetic patients (change, 4 (−17 to 44) %, $P = 0{\cdot}20$). Individual changes in LDL cholesterol, apolipoprotein B and triglycerides were unrelated to changes in PCSK9 ($P > 0{\cdot}10$ for all).

Conclusions: Plasma PCSK9 levels are unaffected by exposure to moderate 24 h hyperinsulinemia in healthy and type 2 diabetic individuals.

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High HDL is not always good

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The risk of cardiovascular disease (CVD) is inversely related to the plasma HDL cholesterol (HDL-C) concentration, and a low HDL-C even predicts CVD risk in statin treated subjects. HDL particles contain a large number of anti-oxidative,

anti-inflammatory and anti-proliferative proteins, which are likely to be responsible for HDL's atheroprotective properties.

HDL has been recognized as a therapeutic target. However, the first drug that elicited a strong increase in HDL-C, the cholesteryl ester transfer protein (CETP) inhibitor, torcetrapib, has failed to ameliorate cardiovascular risk. These negative findings challenge the concept that raising HDL-C is a valid approach to reduce the burden of CVD. In this regard, it is relevant that epidemiological evidence is accumulating that high HDL-C levels do not neccesarily predict reduced CVD risk. The IDEAL trial and the EPIC-Norfolk case-control study demonstrated that (recurrent) CVD risk is not decreased in subjects with the highest HDL cholesterol and the greatest mean HDL particle size. Among participants community-dwelling PREVEND cohort, we were recently able to identify a high HDL-C, high C-reactive protein level subgroup of individuals at increased risk for a first cardiovascular event. Subjects with increased recurrent CVD risk despite high HDL-C have also been noted in THROMBO post-infarction study.

The role of CETP-mediated pathways in the development of atherosclerosis is still controversial. Other factors, such as the cholesterol ester esterifying enzyme, lecithin: cholesterol acyltransferase (LCAT), though playing a beneficial role in the reverse cholesterol transport pathway, could also exert pro-atherogenic properties, particularly in the context of high HDL-C.

Given the clinical relevance to develop novel treatment strategies to combat atherosclerotic diseases, much effort should be paid to identify mechanisms and pathways explaining why high HDL is not always cardioprotective.

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Increased plasma LCAT activity and hyperglycemia contribute to decreased anti-oxidative functionality of HDL in type 2 diabetes mellitus

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Background: The anti-oxidative properties of high density lipoproteins (HDL) are considered to be important for atheroprotection. We determined the extent to which the ability of HDL from T2M patients to protect against low density lipoprotein (LDL) oxidation *in vitro* is impaired, and tested relationships of HDL's anti-oxidative properties with glycaemic control and with HDL-associated proteins [lecithin: cholesterol acyltransferase (LCAT) and paraoxonase-1 (PON-1)].

Materials and methods: In 74 T2DM patients and 75 control subjects, the anti-oxidative ability of HDL was measured as the HDL anti-oxidation capacity (% inhibition of LDL oxidation) and the HDL anti-oxidation index (% LDL oxidation inhibition*HDL cholesterol concentration). LCAT activity was assayed by an exogenous substrate method. PON-1 was measured as its arylesterase activity.

Results: The anti-oxidative capacity of HDL from T2DM patients was similar compared to control HDL. Notably, the HDL anti-oxidation index was decreased in T2DM ($P = 0{\cdot}005$), in conjunction with lower HDL cholesterol ($P < 0{\cdot}001$), increased LCAT activity ($P < 0{\cdot}001$) and decreased PON-1 activity ($P < 0{\cdot}001$). In T2DM, the HDL anti-oxidative capacity was correlated inversely with fasting glucose, HbA_{1c} and LCAT activity ($P < 0{\cdot}01$ to $P < 0{\cdot}001$), whereas the HDL anti-oxidation index was positively related to PON-1 activity ($P < 0{\cdot}10$). Multiple linear regression analysis demonstrated that in the combined subjects both the HDL anti-oxidation capacity ($P < 0{\cdot}05$) and the HDL anti-oxidation index ($P < 0{\cdot}001$) were inversely related to LCAT activity, independently of diabetes status, glycaemic control and PON-1 activity.

Conclusions: The anti-oxidative functionality of HDL is impaired in T2DM taking account of lower HDL cholesterol. In addition to hyperglycaemia, LCAT may impair HDL functionality.

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Large scale quantitative analysis of protein phosphorylation during foam cell formation

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Background: Foam cells are formed from differentiating monocytes accumulating oxidized lipoprotein particles (oxLDL) and represent critical initial steps in development of atherosclerotic lesions. Large scale screening of protein phosphorylation induced by oxLDL was applied to identify regulatory kinases/phosphatases involved in a foam cell formation.

Materials and methods: Human monocytic cell line THP-1 was used as a model for foam cell formation. Cells were treated for 15 min with 12·5 μg mL^{-1} oxLDL, whole cell lysates of control and oxLDL treated cells were enriched for phosphoproteins, digested into peptides and labelled with isobaric labels allowing semi-quantitative analysis of phosphoproteins present using mass spectrometry (MS). Pooled samples were fractionated using strong cation exchange column and directly processed for analysis using LC-MS/MS. Data obtained were calculated for phosphoprotein up-regulation in oxLDL fractions where 1·8-fold increase was determined significant. Up-regulated proteins were further analysed using Ingenuity Pathway Analysis software to visualize protein networks and map biological functions described in literature.

Results: Treatment of THP-1 cell line with oxLDL led to increased phosphorylation of 166 proteins compared to non-treated control. Pathway analysis revealed activation of several regulatory kinases within canonical pathways, such as protein kinase C delta and 1-phosphatidylinositol 3-kinase, which are both involved in oxLDL macropinocytosis.

Conclusions: Phosphoproteomic approach in a combination with isobaric labelling is a new and feasible method that allows large scale based screening of biological targets which will be further studied regarding their role in a foam cell formation.

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Higher glomerular filtration rate predicts lower high density lipoprotein cholesterol and apolipoprotein A-I concentrations in subjects without compromised kidney function

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Background: The kidney contributes to apolipoprotein (apo)A-I catabolism in animal studies probably via dissociation from mature high density lipoproteins (HDL), enabling glomerular passage and subsequent degradation. However, the relationship of HDL with kidney function in subjects without renal insufficiency remains unclear. We tested relationships of HDL cholesterol (HDL-C) and apo-I with kidney function in subjects without severe chronic kidney disease.
Materials and methods: Included was a random sample of the general population (part of the PREVEND cohort). Kidney function [estimated glomerular filtration rate (e-GFR) by two well established equations and creatinine clearance], HDL-C, apoA-I, apoA-II, triglycerides and insulin resistance ($HOMA_{ir}$) were measured in 2484 fasting subjects (e-GFR > 45 mL min^{-1} per 1·73 m^2) without macroalbuminuria, cardiovascular disease, diabetes, or the use of anti-hypertensives and/or lipid lowering agents.
Results: HDL-C ($r = -0{\cdot}056$ to $-0{\cdot}102$, $P < 0{\cdot}01$ to $P < 0{\cdot}001$) and apo A-I ($r = -0{\cdot}096$ to $-0{\cdot}126$, $P < 0{\cdot}001$) were correlated inversely with both GFR estimates and creatinine clearance in univariate analyses. Multiple linear regression analyses also demonstrated inverse relationships of HDL-C and apoA-I with all measures of kidney function even after adjustment for age, sex, waist circumference, $HOMA_{ir}$, triglycerides, and urinary albumin excretion ($P = 0{\cdot}053$ to $0{\cdot}004$). ApoA-II was unrelated to kidney function.
Conclusions: HDL-C and apoA-I are inversely related to e-GFR and creatinine clearance in subjects without severely compromised kidney function. These findings therefore agree with the concept that the kidney contributes to apoA-I regulation in humans. The absence of an association of apoA-II with kidney function supports the concept that this more hydrophpobic apolipoprotein is not filtered by the glomerular membrane. Glomerular hyperfiltration may confer a pro-atherogenic lipoprotein profile.

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Type I diabetes mellitus decreases *in vivo* macrophage-to-feces reverse cholesterol transport despite increased biliary sterol secretion

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Background: The pathophysiology underlying the increased risk for cardiovascular morbidity and mortality in type I diabetes mellitus (T1DM) patients is still incompletely understood. Therefore, our study aimed to delineate the effects of T1DM on hepatic sterol metabolism and reverse cholesterol transport (RCT) *in vivo*.
Materials and methods: T1DM was induced in C57BL/6J mice by a single injection of alloxan, resulting in severely elevated plasma glucose levels from day 2 on ($P < 0{\cdot}05$). Experiments were performed 8–10 days after injection. *In vivo* macrophage-to-feces RCT was studied following intraperitoneal injection of ^{3}H-cholesterol-loaded primary mouse macrophage foam cells.
Results: T1DM resulted in a 2·6-fold higher bile flow ($P < 0{\cdot}05$) and increased biliary secretion rates of bile salts (10·4 fold; $P < 0{\cdot}001$), phospholipids (4·5 fold; $P < 0{\cdot}001$) and cholesterol (5·4 fold; $P < 0{\cdot}05$). Fecal bile acid output was significantly higher in T1DM mice (1·5 fold; $P < 0{\cdot}05$), while mass excretion of fecal neutral sterols remained unchanged. The amount of ^{3}H-cholesterol within plasma tended to be increased 48 h after macrophage injection in T1DM mice ($P = 0{\cdot}07$). Interestingly, experimental T1DM significantly reduced overall RCT reflected by a 20% lower fecal excretion of the macrophage-derived tracer into the feces ($P < 0{\cdot}05$), mainly due to a lower content of label within the bile acid fraction ($P < 0{\cdot}05$). Additional *in vitro* experiments revealed that SR-BI-mediated selective uptake from T1DM HDL is reduced by 41% ($P < 0{\cdot}05$), conceivably due to increased glycation of HDL in T1DM mice (+65%, $P < 0{\cdot}01$).

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HDL as modulator of $LpPLA_2$-mediated CVD risk

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Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a calcium-independent PLA2 that degrades platelet-activating factor and oxidized phospholipids. Such phospholipids are accumulated in the artery wall and play key roles in vascular inflammation. In human plasma Lp-PLA2 is primarily associated with low-density lipoprotein (LDL), whereas a small proportion of circulating enzyme is bound to high-density lipoprotein (HDL). The distribution of Lp-PLA2 between LDL and HDL is altered in several types of dyslipidemias and is also influenced by various hypolipidemic drugs. It has been suggested that the role of plasma Lp-PLA2 in atherosclerosis may depend on the type of lipoprotein particle with which this enzyme is associated. In this regard, data from large Caucasian population studies have shown an independent association between the plasma levels of Lp-PLA2 (which primarily corresponds to the LDL-bound enzyme) and the risk of future cardiovascular events. On the contrary, the HDL-associated Lp-PLA2 (HDL-Lp-PLA2) may substantially contribute to the HDL antiatherogenic activities. Thus, Lp-PLA2 significantly contributes to the HDL-mediated inhibition of cell stimulation induced by oxidized LDL. Furthermore, the HDL-Lp-PLA2 exhibits antiinflammatory and antiatherogenic activities in animal models, *in vivo*. Recently we demonstrated that Lp-PLA2 significantly increases the apoA-I-mediated cholesterol efflux from J774 mouse macrophages in a dose-dependent manner. Furthermore, Lp-PLA2 significantly improves the inhibitory effect of reconstituted HDL particles on ICAM-1 and VCAM-1 expression induced by TNF-α in endothelial cells in culture. On a clinical basis of view, the decrease in the ratio of HDL-Lp-PLA2 to total plasma or to LDL-associated enzyme

observed in patients with dyslipidemias, may be useful as a potential marker of atherogenicity in these subjects.

In conclusion, *in vitro* studies as well as studies in animal models and in humans have shown that in the presence of HDL particles, Lp-PLA2 expresses antiinflammatory and antiatherogenic activities, suggesting that HDL may be an important modulator of Lp-PLA2-mediated cardiovascular risk.

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Genetic variation in *PLTP* and CVD risk

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Phospholipid Transfer Protein (PLTP) facilitates the net phospholipid transfer between plasma lipoproteins and participates in the remodeling of high-density lipoprotein (HDL). Although mouse studies support a proatherogenic role for PLTP, little solid evidence exists with regard to the significance of PLTP for atherosclerosis development in humans.

We aimed to investigate whether genetic variants in *PLTP* (six tagSNPs) underlie altered PLTP activity, hepatic PLTP gene expression, HDL particle level and size, as well as risk of cardiovascular disease (CVD). Two independent studies were used to establish the association with PLTP tag SNPs and plasma PLTP activity and mass. Two SNPs were significantly associated with PLTP activity and mass. A gene score, based on these variants and representing lower PLTP activity and mass, was significantly associated with lower hepatic PLTP gene expression ($P = 3{\cdot}2 \times 10^{-18}$; $n = 957$ liver biopsies) and an increased number of smaller sized HDL particles ($P = 3{\cdot}4 \times 10^{-17}$; EPIC-Norfolk: $n = 3375$). In a total of five CVD case-control studies ($n = 4658$ cases; $n = 11\,459$ controls) the PLTP gene score was associated with a lower CVD risk (per-allele odds ratio 0·94, $P = 1{\cdot}2 \times 10^{-3}$). The odds ratio for the highest vs. the lowest gene score was 0·69 (0·55–0·86), $P = 1{\cdot}0 \times 10^{-3}$.

Conclusion: A PLTP gene score, based on two *PLTP* SNPs that are associated with lower PLTP activity and mass, lower hepatic PLTP gene expression and an increased concentration of smaller-sized HDL particles, is associated with decreased risk of CVD. Likewise, PLTP is a pro-atherogenic protein and may be of interest for future therapeutic intervention.

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Cholesterylester transfer protein (CETP) inhibition

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Low plasma levels of HDL-C are strongly associated with an increase in the risk of coronary artery disease. In addition, animal models with genetic defects in HDL metabolism, causing low HDL, have increased susceptibility for the development of atherosclerosis. Therefore, in addition to apoB-containing lipoproteins (apoB-LP), HDL seem to be an important modulator of atherosclerotic disease. Statins decrease apoB-LP and reduce cardiovascular disease, but there is substantial remaining risk. Obviously HDL appears to be a logical next target.

CETP is a plasma protein with a molecular weight of about 79 kDa. The bulk of plasma CETP is bound to HDL. CETP plays an important role in HDL homeostasis because one of the main actions of CETP is the transfer of cholesterylesters (CE) from HDL to apoB-LP, ultimately resulting in low plasma HDL-C. During this process, VLDL are important acceptors and VLDL-triglycerides are transferred back to HDL. The rate of CE transfer in plasma is therefore strongly influenced by plasma triglyceride levels.

In 2006 a trial assessing clinical outcomes of the CETP inhibitor torcetrapib (T) was aborted after excess deaths and cardiovascular events were present in the T arm of the study. Subsequently it was found that the failure of T may have occurred due to off-target toxicity, e.g. effects on aldosterone synthesis resulting in increased blood pressure. Also it was recognized already earlier that CETP could not only have proatherogenic properties (lowering HDL-C and increasing cholesterol in apoB-LP), but also anti-atherogenic properties, e.g. by functioning in the reverse cholesterol pathway by enhancing the elimination of cholesterol via hepatic LDL and VLDL uptake. Despite these questions two new CETP inhibitors have being tested at present: dalcetrapib (D) and anacetrapib (A). D and A both increase HDL-C and decrease LDL, just like T, but do not seem to have significant effects on aldosterone production. Clinical trials evaluating the effects of D and A on cardiovascular outcome are presently underway (DAL-Outcome and REVEAL trials, respectively). The effects of D and A on HDL-C and LDL levels are promising.

As mentioned above, the rate of CE transfer by CETP is not only dependent on the plasma concentration of active CETP, but also on the composition and concentration of the donor and acceptor lipoproteins. Therefore it is crucial to include the actual lipoprotein profile in the interpretation of the transfer process. The rate of CE transfer by CETP is activated by the elevated plasma concentration of chylomicron (remnants) and VLDL after a fatty meal. CETP increases postprandial hypertriglyceridemia and delays plasma triglyceride clearance in transgenic mice and T decreases the postprandial response after a fatty meal in hyperlipidemic patients. It is unknown whether different CETP inhibitors have specific effects on the postprandial response and on the formation of small-dense LDL. The same is true for effects on the functionality of HDL with respect to the arterial wall, e.g. effects on cholesterol efflux from macrophages, effects on vasodilation, thrombosis, susceptibility to oxidative processes etc.

We have to wait for the results of the ongoing clinical trials with D and A before making up the balance between possible benefits and harms related to pharmacological CETP inhibition, even if these drugs are devoid of adverse off-target effects. The outcome of these trials will influence current thinking about HDL cholesterol raising.

Workshop 7: Early life stress and obesity/ Metabolic syndrome

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Mechanisms of stress and obesity development in children

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Chronically distressed individuals can develop obesity and comorbid states, such as dislipidemia, increased blood pressure, insulin resistance and/or glucose intolerance (clustered as the Metabolic Syndrome). Stress is defined as the disturbance of the complex dynamic equilibrium that all organisms must maintain. It is associated with activation of the Stress System, comprised by the Hypothalamic-pituitary-adrenal Axis and the Arousal/Sympathetic Nervous Systems. The stress system functions both in a baseline circadian fashion and on demand, interacting with other systems of the organism to regulate a variety of behavioral, endocrine, metabolic, immune, and cardiovascular functions. The experience of perceived or real intense and/or chronic stress might lead to several psychopathologic conditions, such as anxiety, depression, posttraumatic stress disorder etc., as well as to somatic consequences, such as obesity and the metabolic syndrome, osteoporosis, and impaired reproductive and immune functions. Developing children and adolescents are particularly vulnerable to the effects of chronic stress. Both behavioral and biologic pathways are involved in the association between chronic stress and obesity in adults and children. Emotional eating, meal patterns, lack of sleep, impulsive behaviors and selection of specific foods often characterize stressed individuals. In addition to specific behaviors, dysregulation of the stress system through increased secretion of cortisol and catecholamines, especially in the evening hours, and in concert with concurrently elevated insulin concentrations, leads to development of central obesity, insulin resistance and the metabolic syndrome. In children, chronic alterations in cortisol secretion may have additional effects on cognition and emotional development, timing of puberty, and final stature.

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Emotional/behavioral disorders and obesity in childhood: neuroendocrine perspective

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Associations between emotional and behavioral disorders and weight status have been reported in adults and children: high percentages of depression and/or anxiety have been shown in obese individuals, and, on the other direction, weight changes is a common characteristic of emotional/behavioral disorders. Though the direction of causality between symptoms of stress and obesity is not always clear, both behavioral and biologic pathways may mediate these relations. Emotional eating, sedentary life-style, lack of sleep, and selection of specific foods often characterize individuals with emotional disorders. In addition, dysregulation of the stress system, through increased secretion of cortisol and catecholamines, leads to development of central obesity and the metabolic syndrome. In developing organisms, chronic alterations in secretion of stress hormones may have additional effects on timing of puberty, cognitive development and physical growth.

In a clinical population of obese children, followed at the outpatient obesity clinic of our Pediatric Department, we found a high percentage of symptoms of anxiety and/or depression: children were 3·6 and 5·2 times more likely of reporting symptoms related to depression and anxiety respectively, compared to the general population. Area under the curve for five diurnal salivary cortisol values was significantly associated with symptoms of trait anxiety in obese children. Among these kids, BMI z-score was higher in those with depression and/or anxiety compared to those free of any symptoms.

Symptoms of Attention Deficit Disorder (ADD) were also higher in this cohort compared to the general population. Children with a high level of ADD symptoms had a higher BMI z-score and a greater number of parameters of Metabolic Syndrome compared to obese children without such symptoms.

In conclusion, investigation of emotional-behavioral symptoms and disorders is essential in understanding and treating childhood obesity.

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Fetal stress, birthweight, and metabolic consequences

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An altered fetal/neonatal environment, such as nutrient insufficiency and stress, may have a 'programming' effect on the metabolism of the fetus or neonate leading to long-term adverse metabolic consequences, obesity and metabolic syndrome. Intrauterine growth restricted (IUGR) infants have lower insulin and leptin and higher cortisol levels at birth, but they present insulin and leptin resistance, dyslipidemia and decreased adiponectin concentrations later in life, along with visceral adiposity even without overweight. However, not only the IUGR state but also high birthweight has been associated with subsequent obesity and metabolic syndrome, especially in children exposed *in utero* to either maternal diabetes or obesity. Moreover, evidence is accumulating that preterm infants are also at risk for the later development of insulin resistance and other components of the metabolic syndrome irrespective of whether they are born small or appropriate for gestational age. Despite reduced total body fat mass at birth, preterm infants present a more central adipose tissue distribution as early as term postconceptional age. In addition, recent evidence and studies from our group have shown that levels of certain metabolism-related peptides including adiponectin, ghrelin, leptin and visfatin differ between preterm and full-term infants. Postnatal weight gain and type of feeding seem to play a critical role in the development of adverse metabolic alterations. Thus, nutrition should be the focus of preventive strategies for better outcomes of these high-risk populations.

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Eating behaviors in children: looking beyond nutrients when investigating diet and obesity-related risk-factors

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Current evidence increasingly confirms that a great part of metabolic, cardiovascular and other complex diseases is attributed to stress. Nutritional and energy balance related factors have been studied either in relation to the stress mechanisms or to the stress metabolic consequences, i.e. obesity and insulin resistance, in young and adult populations. In these studies, nutrition and diet have been traditionally investigated in terms of nutrients and of foods. However, eating is a complex entity consisting of a series of individual behaviors, among others the choice of foods, the organization of food into meals, i.e. the meal patterns, and the conditions preceding, around and following an eating episode, that may multi-dimensionally affect metabolic pathways. In a large pre-adolescent population sample from the area of Athens, we have found that a lifestyle pattern characterized by frequent dinner consumption and cooked meals and well as high vegetables intake was negatively associated with all indices of obesity and insulin resistance. On the other hand in this population sample we have shown that the experience of an important emotional stress, a family divorce, was related to greater body weight and less structured eating and hunger patterns, independent of socio-economic factors. Although this a cross-sectional study, thus not appropriate for establishing cause-effect relationships or elucidating mechanisms, we may speculate that a stress-inducing event has an effect on children's weight status as well as on their dietary habits. In conclusion, looking beyond nutrients and investigating multi-faceted dietary patterns and eating behaviors may be an alternative and/or complementary approach to evaluate diet and obesity-related risk-factors associations.

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Reference curves for fetal biometry in the Greek population: A study of 8500 fetuses

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Background: Growth curves are recommended to derive from National population. This study aims to calculate reference intervals for fetal biometry in the Greek population.
Materials and methods: The study included 8500 fetuses examined in two fetal medicine centers (Athens-Thessaloniki). Gestational age was confirmed in all cases by means of crown-rump length (CRL) measurement during the first trimester, and all ultrasound examinations were performed by operators certified by the Fetal Medicine Foundation. Normal curves were calculated according to the methodology described by Royston and Wright for biparietal diameter (BPD), occipito-frontal diameter (OFD), abdominal circumference (AC) and femoral length (FL). Moreover, the distribution of observations according to our models was compared to the distribution arising from the commonly used formulas of Snijders *et al*. and Chitty *et al*.
Results: The examined biometric parameters were adequately described by the following quadratic equations:

$BPD = -40{\cdot}327 + 5{\cdot}309*GA - 0{\cdot}044*GA^2$ (SD = 2·852), $R^2 = 0{\cdot}967$

$Log_{10}OFD = 0{\cdot}909 + 0{\cdot}059*GA - 7{\cdot}651E{-}4*GA^2$ (SD = 0·016) $R^2 = 0{\cdot}973$

$AC = -101{\cdot}242 + 13{\cdot}672*GA - 0{\cdot}058*GA^2$ (SD = 0·428*GA) $R^2 = 0{\cdot}967$

$FL = -40{\cdot}167 + 4{\cdot}348*GA - 0{\cdot}037*GA^2$ (SD = 1·534) $R^2 = 0{\cdot}977$

Compared to the models according to Snijders *et al*. and Chitty *et al*., the measurements of our population were higher than expected for BPD and AC and lower than expected for FL.
Conclusions: Data from a very large sample of foetuses with confirmed gestational age were used to calculate nomograms for fetal biometry in the Greek population. Compared to the commonly used curves which were calculated for Northern European populations, the distribution of our observations presents small but statistically significant differences.

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Prenatal nutritional stress and postnatal growth in an experimental model

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Background: Prenatal food restriction is associated with low birth-weight of the offspring, whereas postnatal environment further modifies its clinical and metabolic characteristics. The aim of this study is to assess the impact of prenatal nutritional stress and postnatal food manipulation on growth and fat accumulation in Whistar rats.
Materials and methods: Pregnant Wistar rats were obtained at 11 days of gestation. At 14 days of gestation animals were assigned to one of the two following nutritional groups:
(i) Standard diet Group; and (ii) Starved Group: receiving 50% food restricted diet. On postnatal day 26 the litters underwent DEXA assessment to determine body composition. The following groups were studied: A (IUGR/normally fed postnatal), G (Noniugr/normally fed postnatal), B (IUGR/starved postnatal), H (Noniugr/starved postnatal), X (normally fed pre/normally fed postnatal).
Results: Weight at 26 days was comparable between groups G (93·95 ± 14·13) and X (97·44 ± 10·27) while postnatal starvation influenced both groups B (34·76 ± 10·66) and H (38·74 ± 11·02). Abdominal fat percentage (assessed by DEXA) was significantly higher in group G (10·62 ± 21·46, $P = 0{\cdot}01$) compared to the other four groups. Groups A (3·13 ± 2·62) and X (3·09 ± 2·99) were comparable whereas values for group B and H were (1·57 ± 9·01) and (0·02 ± 14·7) respectively.
Conclusion: Offsprings with normal birth weight born of nutritionally stressed mothers who received normal diet postnatally had the higher percentage of abdominal fat compared to the other groups. The stressed non-IUGR groups showed a greater impact of postnatal diet on abdominal fat percentage compared to the stressed IUGR groups, implying that low birthweight may serve as an adaptive phenotype in prenatally stressed rats.

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Cholecystokinin (CCK) deficient mice dysplay abnormal gallbladder hypomotility which affects cholesterol crystallization and growth habits in bile. Relevance for cholesterol gallstones (another fellow traveller with metabolic syndrome)

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Background: The first irreversible physical-chemical step in the formation of cholesterol gallstones is the precipitation of cholesterol crystals from supersaturated bile. How abnormal gallbladder motility influences crystallization and precipitation of excess cholesterol in gallbladder bile is poorly known. The impact of gallbladder dysmotility on cholesterol crystallization and growth habits was studied in the mouse model of cholelithogenesis.

Methods: After cholecystectomy, fresh gallbladder bile was immediately examined for the presence of mucin gels, liquid and solid crystals, and sandy and true gallstones by polarizing phase contrast light microscopy in male CCK (-/-) and (+/+) mice ($n = 4$ per group) before and during feeding a lithogenic diet (15% butterfat, 1% cholesterol and 0·5% cholic acid) for 15 days. Gallbladder size and emptying function were measured (gravity) after an overnight fasting or a high fat meal, respectively. Bile lipid compositions were measured by biochemical methods.

Results: Fasting gallbladder volumes were larger in (-/-) mice than (+/+) mice (50–65 μL vs. 20–30 μL, $P < 0·05$) with either chow or lithogenic diet. Fat-dependent gallbladder emptying was impaired in (-/-) mice. Cholesterol saturation indexes were higher in (-/-) mice than (+/+) mice (1·3 vs. 1·0). During the 15-day feeding of the lithogenic diet, (+/+) mice formed only small amounts of mucin gel and liquid crystals, but not solid cholesterol crystals. In contrast, at day 6, (-/-) mice had formed large amounts of non-birefringent amorphous mucin gels, and aggregated and fused liquid crystals with Maltese-cross birefringence and focal conic texture. At day 9, several small single classic parallelogram-shaped cholesterol monohydrate crystals were detected, embedded in mucin gels. Also, many tubular crystals fractured at ends to produce plate-like cholesterol monohydrate crystals with notched corners. At day 12, three crystal growth habits existed: proportional enlargement patterns, spiral dislocation growth patterns, and twin crystal growth patterns, which induced crystals enlarged in size. Furthermore, aggregated cholesterol monohydrate crystals were surrounded by mucin gels to form disintegratable amorphous sandy stones, with individual solid crystals projecting from the edges. At day 15, 50% of (-/-) mice formed true gallstones.

Conclusions: Absence of CCK impairs gallbladder motility function. Gallbladder size increases leading to stasis and longer residence time of excess cholesterol which ultimately crystallizes. At the early stage of gallstone formation, two pathways of liquid and polymorph anhydrous crystals evolve to triclinical monohydrate crystals with at least three modes for cholesterol crystal growth.

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Predictors of change in BMI z-score from childhood to adolescence in a Greek birth cohort

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Background: Prevention of obesity has focused on childhood as a target period. Our aim was to examine the long-term effects of lifestyle, socioeconomic, and parental parameters on change in BMI from childhood to adolescence in a representative Greek birth cohort.

Population and methods: We used a population-based representative sample of Greek adolescents, followed-up from birth to the ages of 7 and 18 years, to assess influences on trajectories of BMI from childhood (7 years) to adolescence (18 years). We examined the role of peripartum, early development and parental characteristics in predicting change in BMI z-score among boys and girls separately.

Results: Positive change in BMI z-score from 7 to 18 years was greater among boys compared to girls ($P < 0·001$). An increase in BMI z-score was associated with underestimation of body shape among both boys and girls. An increase in BMI z-score was also associated with higher scores on the emotional problems scale (Rutter A2) among boys, whereas an inverse association was found with behavioural problems. Among girls, an increase in the members of family and being choosey about their food in childhood were associated with increasing BMI. Maternal smoking during pregnancy and paternal stress were associated with BMI declines for girls.

Conclusions: Our results suggest that obesity strongly persists from childhood to late adolescence in boys. Attaining healthy eating habits and early recognition of emotional problems in childhood could be powerful tools for reducing obesity. Many children may benefit from building up a positive and correct opinion of their body shape and adopting stress coping strategies.

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Association of Vitamin 25[OH]D3 with metabolic syndrome in young women

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Background: The prevalence of vitamin D insufficiency is high among obese subjects and low serum levels of 25-hydroxyvitamin D (25[OH]D3) have been associated with increased risk of the metabolic syndrome (MS). We aimed to explore whether MS is associated with abnormal serum levels of vitamin 25[OH]D3 in young women with abnormal body weight.

Materials and methods: The study group included 52 young women aged 20–40 years with excessive body mass (BMI ≥ 25 kg m^{-2}). Metabolic syndrome diagnosis based on the definitions of the International Diabetes Federation (IDF 2005) for a Europoid women was made in 27 women. Serum was assayed for HDL-C (HDL-cholesterol), triglycerides (TG), glucose (Architect ci8200, Abbott Diagnostics). Vitamin 25[OH]D3 was assayed using an electrochemiluminescence immunoassay on the Elecsys 2010 (Roche Diagnostics GmbH).

Results: Vitamin D insufficiency (≤ 30 ng mL^{-1}) was present in 40 of the 52 young women (76·9%). Serum vitamin

25[OH]D3 concentrations were lower in women with metabolic syndrome than in women without MS (19·9 ± 9·5 ng mL^{-1} vs. 27·3 ± 11·4 ng mL^{-1}, $P = 0·02$). Concentration of vitamin 25[OH]D3 was the lowest (17·7 ng mL^{-1}) in women having 4–5 features of MS ($n = 6$). Vitamin D insufficiency was more prevalent in women presenting with MS, compared to those who did not achieve the criteria for this syndrome (63% vs. 37% respectively). When serum concentrations of 25[OH]D3 were categorized in tertiles, there was a decreasing prevalence of MS in women with increasing concentrations of vitamin 25[OH]D3 (T1 vitamin 25[OH]D3 < 17·8 ng mL^{-1} – MS 52%; T3 vitamin 25[OH]D3 26·8 ng mL^{-1} – MS 21%).

Conclusions: Vitamin D insufficiency is associated with the metabolic syndrome in young women with excessive body weight.

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Is there any suppressive effect of estrogens on the hypothalamic-pituitary-thyroid axis in overweight and obese girls during and after puberty?

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Background: Several studies have shown that obese individuals may demonstrate moderately increased thyrotropin (TSH) levels, though being euthyroid. The aim of the present study was to assess the set point of hypothalamic-pituitary-thyroid (HPT) axis in overweight and obese children according to their gender and pubertal status.

Methods: Circulating levels of TSH, total triiodothyronine (T_3) and total thyroxine (T_4) were determined in 389 overweight and obese children (173 boys) aged 4–16·5 years (mean age = 9 ·99 years). All children included in the study had a body mass index (BMI) > 1 z-score (mean BMI-z-score = 3·08 ± 1·4 SD), based on the Greek growth curves. Children were divided in four groups according to their gender and pubertal status (prepubertal and pubertal, girls and boys). In each group, a correlation test was performed between TSH and BMI z-score, while mean values of TSH, T4 and T3 were compared between prepubertal and pubertal groups within the same gender.

Results: In both prepubertal girls and boys, a positive correlation was found between TSH and BMI z-scores (Pearson correlation coefficient $r = 0.\ 23$, $P < 0·05$ and $r = 0·31$, $P < 0·001$ respectively), which was not sustained during and after puberty. Interestingly, in pubertal girls TSH, T4 and T3 concentrations were all lower compared to values of prepubertal girls ($P < 0·05$, $P < 0·001$ and $P < 0·002$ respectively) indicating a lower functional set point of HPT axis during and after puberty. In boys no such correlation was found.

Conclusion: We suggest that estrogens and/or other puberty related hormones may exert a suppressive effect on the HPT axis in overweight and obese girls during and after puberty.

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How is the mood of patients with nonalcoholic fatty liver disease?

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Background and aim: Mood may influence health and emotional disorders have been reported as associated with inflammatory states. The purpose of this paper was to evaluate if there was an association between anxiety, depression and non-alcoholic fatty liver disease, in patients with normal and elevated serum transaminases.

Methods: In this study we compared the clinical and biological parameters, the anxiety and depression scores between several groups of patients: a group of 38 female patients with non-alcoholic fatty liver disease (NAFLD), a group of 18 female patients with B or C chronic viral hepatitis, and a group of 24 male patients with NAFLD. Depression was investigated by BDI and anxiety by STAI questionnaires.

Results: Fifty per cent of patients (both with NAFLD and chronic hepatitis) had a depression score suggesting moderate or severe depression. The means of the scores for depression, total distress and anxiety as state were higher in female patients from urban areas. No difference was noticed when comparing the means for depression, anxiety, distress between the group with chronic hepatitis and liver steatosis. There is a strong association between female gender and depression, with a relative risk of 2·81 (95% CI = 1·54–5·12). There were no correlations between the scores for personality traits and serological tests in neither of groups.

Conclusion: Our results do not support any relationship between NAFLD and depression or anxiety. Prospective studies should be done in order to assess the real influence of depression and anxiety on the outcome of patients with NAFLD or chronic viral hepatitis.

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Differences between alcoholic and nonalcoholic fatty liver in respect to emotional distress and Cognitive Coping Mechanisms

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Background and Aim: Patients with liver steatosis may present different emotional disturbances. These can be caused by deficitary coping. The aim of this study was to assess the cognitive coping mechanisms as mediators between automatic thoughts and emotional distress. We looked for two groups of patients with fatty liver: alcoholic and non-alcoholic fatty liver.

Methods: The group studied included 63 patients (mean age 57 years.). We used following questionnaires: Automatic Thoughts Questionnaire, Emotional Distress Profile and Cognitive Coping Mechanisms Scale. For data analysis we used partial correlation coefficient and general linear model.

Results: Our data showed the mediator role of cognitive coping mechanisms ($r = 0·62$, $P < 0·01$). The comparison denotes significant differences between groups with alcoholic and non alcoholic etiologies ($P < 0·01$). Alcoholic groups had higher level of automatic thoughts ($m = 56·6$) and emotional distress ($m = 102·88$) comparative with non alcoholic groups ($m = 42·4$, 74·6), and used mostly coping mechanisms as projection, repression and denial.

Conclusion: The results are useful for developing a cognitive intervention model for patients with hepatic steatosis in clinical setting with the aim of reducing patients's emotional distress.

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Circulating lipoprotein-associated phospholipase A2 levels are elevated in obese children

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Background: Obesity and cardiovascular disease are often comorbid, but the pathophysiologic mechanisms that link the two are not fully understood. Lipoprotein-associated phospholipase A2 (Lp-PLA2) was recently found to be predictive of thromboembolic episodes in adults. The aim of this study was to evaluate the function of this lipase and its importance in children, especially obese ones.
Materials and methods: Sixty-seven lean (39 boys – 28 girls, mean BMI z-score: −0·23 ± 0·78) and 66 obese (32 boys – 34 girls, mean BMI z-score: 4·39 ± 1·18) age-matched ($P = 0·08$) children, aged 6–12 years, were studied. All children had a physical examination and morning blood drawn after 12 h of fasting. Glucose, insulin, lipid profile and Lp-PLA2 were determined. Plasma concentrations of Lp-PLA2 were determined by an enzyme-linked immunosorbent assay (ELISA) kit (PLAC Test). BMI z-score was calculated and children were categorized as obese according to the Cole criteria.
Results: Plasma Lp-PLA2 levels were significantly higher in obese children (322·5 ± 77·8 ng mL^{-1}) than those of the normal weight group (278·0 ± 64·4 ng mL^{-1}), ($P < 0·00001$). Lp-PLA2 concentrations were significantly correlated with the BMI z-score ($P = 0·006$).
Conclusions: We found significantly higher Lp-PLA2 levels in obese children than lean controls. Interestingly, they all had levels > 200 ng mL^{-1}, which are thought to correlate with atherosclerosis and a high thromboembolic risk in adults. The positive correlation of Lp-PLA2 with BMI reveals an effect of obesity on this marker, which suggests vascular involvement caused by the increase of weight, even at a very young age. More prospective studies should be done in order to evaluate whether Lp-PLA2 could be added to the panel of tests used to identify young individuals at high CVD risk.

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Subclinical inflammation, the metabolic syndrome, and coronary atherosclerosis in men and women

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Background: The metabolic syndrome (MetS) and stable coronary artery disease (CAD) frequently coincide; the contributions of these entities to subclinical inflammation are unknown.
Materials and methods: We enrolled 1012 consecutive patients, 656 men and 356 women, undergoing coronary angiography. The MetS was defined according to the AHA revision of the NCEP ATP-III criteria; coronary stenoses ≥ 50% were considered significant.
Results: Serum concentrations of CRP were significantly higher in patients with the MetS compared to subjects without both in men (0·47 ± 0·68 mg dl^{-1} vs. 0·36 ± 0·51 mg dl^{-1}; $P < 0·001$) and in women (0·44 ± 0·52 mg dl^{-1} vs. 0·33 ± 0·44 mg dl^{-1}; $P = 0·005$). In contrast, CRP did not differ significantly between patients with significant CAD and those who did not have significant CAD in either gender ($P = 0·105$ for women and $P = 0·461$ for men). When all MetS traits were entered simultaneously into one ANCOVA model, in men only the low HDL-C criterion proved independently associated with CRP ($F = 36·65$; $P < 0·001$), whereas in women the low HDL-C and the high glucose criteria significantly predicted serum CRP after multivariable adjustment ($F = 5·55$; $P = 0·019$ and $F = 5·31$; $P = 0·022$, respectively).
Conclusions: CRP is strongly associated with the MetS but not with angiographically diagnosed coronary atherosclerosis in men and women. The overall association of the MetS with subclinical inflammation both men and women is driven by the low HDL cholesterol feature and in women additionally by the high glucose feature.

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An obesity preventive score is negatively associated with obesity indices and biomarkers of insulin resistance: results from the GENDAI cohort

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Background: Eating behavior is a complex entity consisting of multiple behaviors, which include food choice, meal patterns as well as conditions preceding, around, and after eating episodes. Most of these behaviors have been investigated either individually in relation to overweight and metabolic risk factors in children and adolescents or with physical activity. For this purpose the aims of the present study were (i) to calculate a score comprising of a series of eating and physical activity behaviors; and (ii) to investigate how it affects anthropometric and biochemical markers associated with obesity and insulin resistance indexes.
Materials and methods: In this cross-sectional study, 1138 healthy school-aged children (53% girls; age: 11·2 ± 0·7 years) participated. Detailed dietary, behavioral, lifestyle, anthropometric and biochemical variables were recorded for participants. An overweight/obesity preventive score comprising of eight target lifestyle behaviors, based on the recommendations of the Expert Committee of American Academy of Pediatrics, was calculated for all subjects.
Results: Overweight/obesity preventive score was significantly negatively associated with obesity indices, namely body mass index ($P = 0·001$), waist circumference ($P = 0·002$), triceps ($P = 0·001$) and subscupular ($P = 0·009$) skinfolds, and total body fat ($P = 0·002$), after adjustment for potential confounders. A significant negative association was also observed between the overweight/obesity preventive score and several biomarkers, such as serum insulin ($P = 0·004$), leptin ($P = 0·003$) and the HOMA-IR index ($P = 0·007$), following adjustment for confounding factors.
Conclusions: Our study shows that higher adherence to an overweight-obesity preventive diet and physical activity pattern is associated with lower values in several obesity- and

insulin resistance-related biomarkers, indicating that the adoption of healthy eating and activity habits protects children from overweight and it is also associated with better glucose homeostasis.

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Obesity and associated cardiovascular risk factors among Greek adolescents in a tertiary adolescent center

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Background: The purpose of this study is to examine the prevalence of obesity and cardiovascular risk factors among adolescents presenting in a tertiary unit.
Materials and methods: Biochemical parameters were evaluated in 428 adolescents (age range 11–18 years, mean 14·4 years). Adolescents were categorized according to body mass index (BMI) as underweight (BMI < 5th percentile), healthy (BMI 5th–84thpercentile), overweight (BMI 85th–94th percentile) or obese (BMI ≥ 95th percentile).

Dyslipidemia was assessed based on cutoff points for elevated low-density lipoprotein cholesterol LDL-C (≥ 130 mg dl^{-1}), low high-density lipoprotein cholesterol HDL-C (≤ 35 mg dl^{-1}), high total cholesterol TC (≥ 200 mg dl^{-1}) and high triglyceride levels (≥ 150 mg dl^{-1}). The levels of fasting serum glucose and insulin were also measured.
Results: Almost one third (29·6%) of the adolescents studied were overweight, whereas 15·5% were obese. 9·3% of the participants had fasting glucose levels of 100 mg dL^{-1} or above and 17·6% had fasting insulin levels > 25 μIU mL^{-1}. Hypercholesterolemia, high LDL-C, low HDL-C and hypertriglyceridemia were noted in 11·9%, 8·3%, 4·6% and 5·4% of adolescents respectively.
Conclusions: This ongoing study underscores the importance of identifying and screening youth particularly with regard to obesity and cardiovascular risk factors. Surveillance data can aid public health lifestyle programs and interventions.

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Birth weight reference curves at 32–41 weeks of gestation with confirmed gestational age: association with maternal obesity and diabetes

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Background: Growth curves are recommended to derive from National population and birthweight can be influenced by maternal disease. This study aims to calculate birth weight reference curves of neonates with confirmed gestational age of delivery by ultrasound in the Greek population and to assess the impact of maternal obesity and diabetes on birth weight.
Materials and methods: The study included 587 fetuses-neonates whose gestational age was confirmed by ultrasound at 11–13 weeks of gestation by ultrasound. Based on Royston and Wright methodology we calculated birth weight curves in relationship with gestational age. Furthermore using linear regression analysis for z-scores we assessed the association of birth weight with fetal sex, maternal age, maternal BMI, obesity and gestational diabetes.
Results: The relation between birthweight (BW) and gestational age (GA) is described by the following equation: $Log_{10}BW = -4{\cdot}0808 + 0{\cdot}3659*GA - 0{\cdot}0043*GA^2$ ($R^2 = 0{\cdot}374$). The respective equation for boys is: $Log_{10}BW = -1{\cdot}2837 + 0{\cdot}2231*GA - 0{\cdot}0025*GA^2$ ($R^2 = 0{\cdot}326$) whereas for girls is $Log_{10}BW = -8{\cdot}1402 + 0{\cdot}5752*GA - 0{\cdot}0070*GA^2$ $R^2 = 0{\cdot}436$). Statistically significant parameters affecting birthweight are fetal gender ($P = 0{\cdot}001$) and gestational diabetes ($P = 0{\cdot}022$).
Conclusion: data analysis of fetuses with confirmed gestational age of delivery by ultrasound in the Greek population showed that the mean birthweight at 38, 39 and 40 weeks of gestation are 3018, 3215 and 3358 g respectively and is expected to be higher in males and in pregnancies complicated by diabetes.

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Improved on soluble vascular cell adhesion molecule (VCAM-1) after a short physical exercise program in young male adults diagnosed with metabolic syndrome

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Background: It is widely accepted endothelial dysfunction may play an important role during the development of atherosclerotic plaque. Recent studies have reported regular exercise may reduce proinflammatory biomarkers in patients with metabolic syndrome However to date little information is available on the influence of exercise on levels of endothelial adhesion molecules. This study was designed to determine the influence of exercise on soluble vascular cell adhesión molecule (VCAM-1) in young male adults with metabolic syndrome.
Materials and method: Sixty adult men with metabolic syndrome according to the criteria reported by the National Cholesterol Education Program Adult Treatment Panel III volunteered for this study. Forty-five were randomly included in experimental group to perform a 6-week aerobic training program, 3 days per week, consisting of warm up (10-min), main part [20–35-min (increasing 5 minutes each 3 weeks)] at a work intensity of 60–75% of peak heart rate (increasing 5% each 2 weeks) and cool-down (10-min). Control group included 15 age, sex and BMI-matched men with metabolic syndrome that will not perform any program. Further our protocol was approved by an institucional ethic committee. Serum soluble VCAM-1 concentration was measured by ELISA, using a commercially available kit (Parameter; R&D Systems) twice: 72-h before starting the program and after its ending.
Results: When compared to baseline soluble VCAM-1, concentration was decreased significantly after our 6-week protocol ($453{\cdot}9 \pm 17{\cdot}6$ ng mL^{-1} vs. $372{\cdot}8 \pm 18{\cdot}6$ ng mL^{-1}; $P < 0{\cdot}05$). No changes were reported in controls.
Conclusion: A 6-week training program decreased soluble VCAM-1 concentration in young male adults with metabolic syndrome.

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Low-density-lipoprotein oxidation was reduced by aerobic training in adult women with metabolic syndrome

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Background: Recent studies have reported oxidative stress may be considered a potential therapeutic target in metabolic syndrome. Fortunately we have recently found aerobic training improved plasmatic total antioxidant status in this group. This study was designed to assess the influence of a 12-week aerobic training program in protein oxidation in women with metabolic syndrome.
Materials and methods: Sixty young women (39·1 ± 3·5 years; 28·7 ± 1·9 kg m^{-2}) with metabolic syndrome according to the criteria reported by the National Cholesterol Education Program Adult Treatment Panel III volunteered for this study. Forty-five were randomly included in experimental group to perform a 12-week aerobic training program, 3 days per week, consisting of warm up (10-min), main part in a treadmill [20–35-min (increasing 5 min each 3 weeks)] at a work intensity of 60–75% of peak heart rate (increasing 5% each 3 weeks) and cool-down (10-min). Control group included 15 age, sex and BMI-matched women with metabolic syndrome that did not perform any training program. Plasma ox-LDL levels were measured using a competitive ELISA (Mercodia, Uppsala, Sweden) that utilizes the murine monoclonal antibody 4E6. This antibody is directed against a conformational epitope in LDL that is generated from substitution of at least 60 lysine residues in apoB with aldehydes.
Results: When compared to baseline, plasma ox-LDL was reduced significantly after a 12-week aerobic training protocol (171·3 ± 12·8 UI vs.130·4 ± 12·0 UI; $P = 0·027$). In contrast, no changes were reported in controls.
Conclusion: Aerobic training reduced LDL-oxidation in women with metabolic syndrome. Further long-term well-conducted studies are required in order to highlight potential clinical benefits of this improvement in these patients.

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Frequent androgen deficiency in infertile men with non-obstructive azoospermia associated with dyslipidemia

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Background: May with a non-obstructive azoospermia (NOA) may be at increased risk of hypogonadism which may subsequently lead to the development of metabolic syndrome, diabetes mellitus type 2, cardiovascular disease and/or osteoporosis. Apart from the aetiological link between NOA and male hypogonadism, testicular sperm extraction (TESE), may further aggravate Leydig cell dysfunction.
Methods: Fasting morning blood samples were obtained for analysis of testosterone, LH, SHBG, total cholesterol, LDL, HDL and triglycerides from 65 men (mean age 35·9 years) who had undergone conventional TESE due to NOA. Blood sampling was done, on average, 2·3 (SD: ± 1·6) years after TESE. Hormone levels were compared with those in 141 proven fertile controls. For 45 of the NOA-patients, pre-TESE morning hormone values were available. Hypogonadism was defined as ongoing androgen replacement therapy or testosterone ≤ 10 nM and/or LH ≥ 10 IU L^{-1}.
Results: Hypogonadism was found in 47% of the patients. The post-TESE odds ratio for hypogonadism was 20 (95% CI 7·9–52) as compared to fertile controls. Pre- and post-TESE testosterone concentrations did not differ significantly (mean ± SD: 14·1 ± 4·9 nM vs. 14·6 ± 4·1 nM; $P = 0·12$) whereas the LH levels increased significantly post-TESE (9·2 ± 8·8 IU L^{-1} vs. 7·9 ± 6·2 IU L^{-1}). Hypogonadal, not androgen treated NOA-men, tended ($P = 0·09$) to have a higher LDL/HDL ratio than infertile men with testosterone values within the normal range.
Conclusion: Men with NOA are at very high risk of androgen deficiency, which even in young subjects can be associated with dyslipidemia. Medical management of these men should include not only sperm extraction, but also endocrine evaluation and follow up.

Workshop 9: Insulin resistance and cardiovascular disease

901
Insulin resistance: pathophysiology

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Insulin resistance denotes reduced sensitivity to the metabolic actions of insulin on its target organs, and is the underlying characteristic of the Insulin Resistance or Metabolic Syndrome.

The role of insulin is storage of energy after food intake in adipose tissue as triglycerides and in the liver and muscle as glycogen. The stored energy can then be mobilized to provide energy substrates (FFAs and glucose) during fasting or in catabolic states such as infection, trauma or stress, when the action of insulin must be temporarily switched off (insulin resistance).

At the cellular level, insulin action is mediated by tyrosine phosphorylation of insulin receptor substrates (IRS) and subsequent activation of the PI3K/Akt signaling pathway. In catabolic states negative regulation of insulin action is induced by phosphorylation of IRS on serine residues by certain intracellular serine kinases, thus preventing tyrosine phosphorylation by insulin. These serine kinases, including JNK, IKK, PKC, are activated by proinflammatory cytokines and stress hormones, released during infection on stress.

It is argued that the mechanism of insulin resistance is inappropriately activated on a chronic basis as a consequence of our modern lifestyle, leading to the manifestations of the Metabolic Syndrome. Current lifestyle, characterized by excess nutrient intake, lack of physical activity and chronic stress, favours the storage of excess fat in visceral fat depots in individuals with genetic predisposition for central fat distribution. Visceral obesity is associated with chronic low-grade inflammation and adipocyte dysfunction with lipid overflow to peripheral tissues. In turn, proinflammatory cytokines and lipid metabolites, accumulated in liver and muscle cells, cause insulin resistance, as would occur in the case of infection or stress. The ensuing insulin resistance leads ultimately to type 2 diabetes and cardiovascular disease.

Thus, insulin resistance could be viewed as an adaptive mechanism that turns maladaptive in the current in environment.

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Effects of insulin resistance on the cardiovascular system

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Insulin resistance (IR) is a metabolic abnormality linked to the development of type 2 diabetes mellitus (DM2) and increased cardiovascular disease (CVD) risk. Other conditions such as impaired glucose tolerance (IGT), the metabolic syndrome and the polycystic ovary syndrome, both linked with abdominal obesity, are also characterized by IR.

Multiple prospective studies have documented an association between IR and accelerated atherosclerosis and CVD: increased risk of myocardial infarction, stroke, hypertension, chronic kidney disease, other CVD and mortality. A cluster of metabolic and cardiovascular disorders such as dyslipidaemia, hypertension, visceral obesity, glucose intolerance, and endothelial dysfunction, each of which is an independent risk factor for CVD, is observed in association with IR; this is also known as cardiometabolic syndrome.

Various complex inter-related pathophysiologic mechanisms may explain the accelerated rate of atherosclerosis associated with IR. The molecular causes of IR, i.e. impaired insulin signaling through the phosphoinositol-3 kinase pathway with intact signaling through the mitogen-activated protein kinase pathway, lead to impaired insulin-stimulated glucose metabolism and reduced glucose tissue uptake, while endothelial nitric oxide (NO)-mediated vasodilation, inhibition of platelet aggregation and vascular smooth muscle cell growth are also impaired, contributing to endothelial dysfunction and atherogenesis. Alterations in adipose tissue fatty acid and adipokine metabolism also play a role; excessive fatty acid release into the bloodstream impairs the ability of insulin to stimulate muscle glucose uptake and to suppress hepatic glucose production, leading to hyperglycemia, while increased free fatty acid delivery to the liver stimulates hepatic very low-density lipoprotein triglyceride production, leading to dyslipidaemia. Noninfectious systemic chronic inflammation associated with visceral obesity and adipose tissue macrophage cytokine production such as TNF-alpha, FFA, IL-1, IL-6, leptin and resistin is also involved. IR also stimulates the production of CRP and PAI-1, both markers of inflammation but also potential mediators of the atherosclerotic process.

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Insulin resistance in women: polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is a common condition of young premenopausal women characterized by both reproductive and metabolic features. Insulin resistance and hyperandrogenism are two inter-related key factors in the pathophysiology of the syndrome and are associated with the presence of cardiovascular risk factors such as dyslipidemia, type 2 diabetes and obesity, even at a young age. Insulin resistance and secondary hyperinsulinemia affect approximately 65–70% of women with PCOS. Many of these women are also obese, which further exacerbates their insulin resistance. PCOS may be related to adverse cardiovascular prognosis although data on clinical cardiovascular endpoints are limited and inconsistent.

Endothelial dysfunction presents early in the atherosclerotic process long before structural vascular lesions occur. We have shown that women with PCOS have significant endothelial dysfunction at an early age (i.e. early 20s), which appears to be largely independent of obesity. In these young women, treatment with metformin or pioglitazone for 6 months induces a similar beneficial effect on endothelial function, which may be attributed to an improvement in insulin

resistance. In addition, in young non-obese women with PCOS, treatment with combination of estrogen and antiandrogen for 6 months appears also to improve endothelial function, mainly due to an improvement in hyperandrogenism. Further research is needed to investigate whether women with PCOS may gain particular benefit from treatment-induced improvement in endothelial function, by either insulin sensitizers or oral contraceptives, and may effectively reduce their cardiovascular risk.

904
Insulin sensitizers: cardiovascular effects

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Cardiovascular disease (CVD) remains the primary cause of mortality for patients with type 2 diabetes mellitus (DM2), which is characterized by insulin resistance (IR). Interventions targeting multiple CVD risk factors such as dyslipidaemia or hypertension have been shown to reduce CVD risk in DM2, while reduction in IR has also emerged as a potential target.

Besides pharmacological interventions, implementation of a regular exercise and diet program may decrease IR, improve glycaemic control and CVD prognosis in DM2patients. Metformin is a biguanide that enhances hepatic insulin sensitivity, improving the effectiveness of insulin in suppressing excess hepatic glucose production, especially in the postprandial state. Decreased gastrointestinal glucose absorption, increased insulin sensitivity in peripheral tissues, and enhanced GLP-1 synthesis may have minor roles. Metformin also has beneficial effects on other IR-related metabolic components including mild-moderate weight loss, improved lipid profile and fibrinolysis and has been demonstrated to reduce CVD risk in overweight DM2 patients.

Thiazolidinediones (TZDs) or glitazones improve insulin sensitivity, acting as nuclear peroxisome proliferator-activated receptor-gamma (PPAR-gamma) agonists that regulate gene transcription involved in adipocyte differentiation, glucose and lipid metabolism. The currently available TZDs, pioglitazone and rosiglitazone, have shown similar beneficial effects on insulin sensitivity and glycemic control, and similar adverse effects on weight gain, fluid retention, risk of heart failure, leg and forearm fractures. Although beneficial effects on surrogate end-points of atherosclerosis have been shown for both, differential effects on CVD risk have been reported; pioglitazone has a beneficial effect, while rosiglitazone appears to increase CVD risk. The differential effect on lipid profile may be the main factor underlying the superiority of pioglitazone in reducing CVD risk. Furthermore, glitazones have also shown beneficial anti-atherosclerotic effects in non-diabetic individuals with IR such as patients with obesity, impaired fasting glucose or impaired glucose tolerance and women with polycystic ovary syndrome.

905
The global nutritional characterization of patients with non-alcoholic fatty liver disease: from factors that may influence their dietary intake to body composition

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Background: Non-alcoholic fatty liver diseases (NAFLD) are recognized in patients with a high body mass index (BMI) and visceral adiposity. Rarely these patients were assessed in the global their nutritional aspects, from dietary intake to total body composition.

Materials and methods: This study evaluated dietary intake and plasma levels of some gastrointestinal peptides that regulate the choice of foods, as well as the global nutritional assessment in 25 patients with NAFLD compared with 16 healthy controls. In all subjects foods intake was evaluated by electronic program (WinFood, Medimatica s.r.l.). The serum levels of Y polypeptide (PYY), pancreatic polypeptide (PP), glucagon-like peptide (GLP)-1, oxyntomodulin (OXY), ghrelin (GHR), orexin (ORE) and cholecystokinin (CCK) were determined by enzymatic immunoassay. Body composition was evaluated by impedenziometric analysis (BIA 101S).

Results: No statistically significant differences were observed for gender, and therefore data are reported globally. Table 1 summarizes only data significantly different between controls and NAFLD patients.

Conclusions: Data indicate that, other than for body composition, NAFLD patients differ from healthy subjects also for their alimentary intake. In this puzzle, it is possible that some gastrointestinal peptides play a fundamental role.

Table 1 Cycles' characteristics by stimulation protocol in relation with AFC categories

	NAFLD	Controls	*P*
BMI (kg m^{-2})	31·1 ± 3·7	24·5 ± 1·4	0·001
Free fat mass%	71·18 ± 4·7	80·5 ± 5·5	0·002
Fat mass%	28·82 ± 4·7	19·5 ± 5·4	0·002
Trunk fat%	36·28 ± 3·9	27·84 ± 2·73	0·005
Lipids (g)	72·9 ± 21·037	47·75 ± 7·76	0·006
Carbohydrates (g)	324·38 ± 87·15	231·2 ± 76·31	0·050
Monounsaturated fatty acids (g)	39·7 ± 15·889	19·38 ± 5·63	0·003
GHR (ng mL^{-1})	75·1 ± 26·36	85·4 ± 23·56	0·050
GLP–1 (ng mL^{-1})	40·2 ± 19·70	49·4 ± 12·4	0·050

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Metabolic and anti-inflammatory benefits of eccentric endurance exercise

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Background: Eccentric endurance exercise (e.g. hiking downwards) is less strenuous than concentric exercise (e.g. hiking upwards) but its metabolic effects are largely unknown.

Material and methods: We allocated 93 healthy sedentary individuals to an exercise intervention program, consisting of hiking downwards a pre-defined route in the Austrian Alps over two months. For the opposite way, a cable car was used where compliance was recorded electronically. The difference in altitude was 540 m; the distance was covered three to five times a week. A matched group of 25 individuals served as a control group.
Results: Compared with baseline, eccentric exercise significantly lowered fasting glucose (97 ± 15 mg dL^{-1} vs. 94 ± 9 mg dL^{-1}; $P = 0{\cdot}025$) and glucose tolerance (239 ± 50 mg*dL^{-1} h^{-1} vs. 217 ± 47 mg*dL^{-1} h^{-1}, whereas both were unchanged in the control group. Body mass index (27·7 ± 4·4 kg m^{-2} vs. 27·4 ± 4·3 kg m^{-2}; $P = 0{\cdot}003$) and C-reactive protein (0·27 ± 0·42 mg dL^{-1} vs. 0·23 ± 0·25 mg dL^{-1}; $P = 0{\cdot}031$) also significantly declined in the eccentric exercise group but not in the control group. Furthermore, eccentric exercise significantly lowered triglyceride tolerance (1959 ± 1330 mg*dL^{-1} h^{-1} vs. 1670 ± 1085 mg*dL^{-1} h^{*-1}; $P = 0{\cdot}003$) and the postprandial leukocyte count (68·8 ± 11·6 G*L^{-1} h^{-1} vs. 66·5 ± 13·6 G*L^{-1} h^{-1}; $P = 0{\cdot}031$), whereas both were unchanged in the control group.
Conclusions: Eccentric exercise is a promising new exercise modality with favourable metabolic and anti-inflammatory effects.

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Factors predicting cardiovascular events in statin-treated diabetic and non-diabetic coronary patients: a prospective cohort study

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Background: We aimed at identifying which lipid factors drive vascular risk in statin treated patients with coronary artery disease (CAD).
Materials and methods: We recorded vascular events over a mean period of 7·2 years in 491 consecutive statin-treated patients with angiographically proven stable CAD, covering 3518 patient-years.
Results: In the total population, low HDL cholesterol [standardized adjusted HR 0·80 (0·67–0·94); $P = 0{\cdot}009$], low apolipoprotein A1 [0·84 (0·72–0·98); $P = 0{\cdot}022$], a small LDL particle diameter [0·84 (0·72–0·98); $P = 0{\cdot}023$], and high triglycerides [1·18 (1·04–1·35); $P = 0{\cdot}013$] predicted vascular events, but not total cholesterol, LDL cholesterol, or apolipoprotein B. Factor analysis in the lipid profiles of our patients revealed an HDL-related factor and an LDL-related factor. Concordant with the results for individual lipid parameters, the HDL-related factor [0·76 (0·65–0·90); $P = 0{\cdot}001$] but not the LDL-related factor ($P = 0{\cdot}644$) predicted vascular events. Patients with type 2 diabetes (T2DM; $n = 116$) were at a higher vascular risk than non-diabetic subjects (52·6% vs. 36·8%; $P = 0{\cdot}002$), and like in the total population the HDL-related factor [0·63 (0·49–0·81); $P < 0{\cdot}001$] but not the LDL-related factor ($P = 0{\cdot}976$) predicted vascular risk in diabetic patients.
Conclusions: The pattern of low HDL cholesterol, low apolipoprotein A1, small LDL particles, and high triglycerides drives vascular risk in statin-treated coronary patients, particularly in those with T2DM.

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Type 2 diabetes significantly modulates the impact of low left ventricular ejection fraction on the risk of cardiovascular events

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Background: We aimed at prospectively investigating the impact of the left ventricular ejection fraction (LVEF) and of angiographically verified coronary artery disease (CAD) on the risk of cardiovascular events in patients with type 2 diabetes (T2DM) and in non-diabetic subjects.
Materials and methods: Cardiovascular events were recorded over 8 years in 629 patients undergoing coronary angiography for the evaluation of CAD. Significant CAD was diagnosed in the presence of significant coronary stenoses with lumen narrowing ≥ 50%, and the baseline LVEF was determined ventriculography.
Results: Prevalence of significant CAD was higher (68·6% vs. 55·5%; $P = 0{\cdot}006$) in patients with T2DM than in non-diabetic subjects; LVEF was similar in these subgroups ($P = 0{\cdot}253$). Prospectively, CAD [HR = 2·07 (1·50–2·88); $P < 0{\cdot}001$] and the LVEF [standardised HR = 0·79 (0·71–0·88); $P < 0{\cdot}001$] both proved predictive of cardiovascular events in an independent manner. The LVEF predicted cardiovascular events in non-diabetic subjects [HR = 0·72 (0·62–0·82); $P < 0{\cdot}001$] but not in patients with T2DM ($P = 0{\cdot}711$). An interaction term LVEF*T2DM was significant ($P = 0{\cdot}047$), indicating that the risk conferred by a low LVEF was significantly higher in non-diabetic subjects than in patients with T2DM. The presence of significant CAD proved predictive of vascular events both in non-diabetic subjects and in patients with T2DM [HRs 1·84 (1·26–2·67); $P = 0{\cdot}001$ and 2·45 91·18–5·06); $P = 0{\cdot}016$, respectively].
Conclusions: T2DM significantly modulates the cardiovascular risk conferred by a low ejection fraction.

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Chronic prednisolone treatment leads to dyslipidemia in mice carrying a dimerization-defective glucocorticoid receptor

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Background: Chronic use of anti-inflammatory synthetic glucocorticoids cause severe side effects, such as insulin resistance and dyslipidemia, which resemble features of the metabolic syndrome. Since glucocorticoids act via the glucocorticoid receptor (GR) both as dimer or monomer, we aimed to elucidate the different effects of chronic prednisolone treatment on metabolic profiles in mice carrying a dimerization-defective glucocorticoid receptor and wild type mice.
Methods: WT and GRdim mice received sustained treatment of prednisolone for 6 days, by implantation of a pellet. Before

and during the treatment, plasma glucose levels and lipoprotein profiles were determined. After 6 days, livers were collected and analyzed for lipid levels and gene-expression pattern.

Results: Six-day prednisolone treatment did not influence fasting glucose levels in GRdim mice, but strongly increased plasma triglyceride and cholesterol levels. This was already apparent after one day of treatment. Prednisolone treatment resulted in a dramatic increase of triglycerides (644 nmol per fraction vs. 72 nmol per fraction, GRdim Pred vs. WT ctr), phospholipids (196 nmol per fraction vs. 15 nmol per fraction) and free cholesterol (201 nmol per fraction vs. 34 nmol per fraction) in the VLDL fraction of prednisolone treated GRdim mice. Surprisingly, hepatic lipid levels did not change. The unusual lipid profile could not be caused by cholestasis since plasma bile acid concentration was not increased. Hepatic gene expression levels showed no major differences in genes involved in cholesterol conversion and HDL metabolism as well as in bile acid secretion and production.

Conclusion: Prednisolone treatment in GRdim mice leads to an atypical VLDL particle containing high levels of triglyceride, phospholipids and free cholesterol. These results indicate an important role of monomeric GR in lipid metabolism and this could contribute to unravelling the mechanisms underlying glucocortoid-induced side effects.

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Insulin resistance is associated with metabolic syndrome but not with angiographically determined coronary artery disease in female patients

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Background: Insulin resistance (IR) is the key feature of the metabolic syndrome (MetS) and in prospective studies predicts atherothrombotic events. Its association with directly visualised coronary atherosclerosis, especially in female patients, is unclear.

Materials and methods: We enrolled 354 female patients undergoing coronary angiography for the evaluation of stable CAD; significant CAD was diagnosed in the presence of coronary stenoses ≥ 50%. IR was determined by the HOMA index; the MetS was defined according to ATPIII criteria.

Results: HOMA-IR scores were significantly higher in MetS patients than in subjects without the MetS (4·9 ± 4·7 vs. 1·9 ± 1·1; $P < 0·001$). HOMA-IR did not differ significantly between patients with significant CAD and those who did not have significant CAD 3·3 ± 3 vs. 3·1 ± 3; $P = 0·823$). When both, the MetS and significant CAD were considered, HOMA-IR was significantly higher in patients with the MetS both among those who had CAD (4·9 ± 4·8 vs. 1·9 ± 1·1; $P < 0·001$) and among those who did not have CAD (5·0 ± 4·7 vs. 1·9 ± 1·1; $P < 0·001$) whereas it did not differ significantly between patients with significant CAD and subjects without significant CAD in patients with the MetS ($P = 0·383$) nor in those without the MetS ($P = 0·860$). Similar results were obtained with the IDF definition of the metabolic syndrome.

Conclusion: In female patients IR is significantly associated with the MetS but not with angiographically determined coronary atherosclerosis.

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Albuminuria is associated with coronary atherosclerosis both in patients with type 2 diabetes and in non-diabetic individuals

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Background: Albuminuria is associated with atherothrombotic events and all-cause mortality. However, it is not known whether albuminuria is associated with directly visualised atherosclerosis.

Materials and methods: We enrolled 909 consecutive Caucasian patients, including 226 patients with type 2 diabetes (T2DM) and 683 non-diabetic subjects referred to coronary angiography for the evaluation of coronary artery disease (CAD). Elevated urinary albumin excretion (UAE) was defined as an urinary albumin to creatinine ratio (ACR) ≥ 30 μg mg^{-1}; significant CAD was diagnosed in the presence of coronary artery lumen narrowing ≥ 50%.

Results: The prevalence of significant CAD was significantly higher in patients with an elevated UAE than in those with normal UAE (65·9% vs. 51·4%; $P < 0·001$). Fully adjusted logistic regression analysis confirmed elevated UAE as a significant predictor of angiographically determined CAD [OR = 1·68 (1·15–2·44); $P = 0·007$]. Similarly, the ACR was significantly associated with significant CAD when treated as a continuous variable [standardized adjusted OR = 1·45 (1·13–1·86); $P = 0·004$]. Concordantly, the ACR proved significantly predictive of significant CAD both in patients with T2DM [1·66 (1·01–2·74); $P = 0·045$] and in patients without diabetes [1·42 (1·05–1·92); $P = 0·023$] in a fully adjusted model.

Conclusions: In conclusion, an elevated UAE is strongly associated with angiographically determined coronary atherosclerosis both in patients with T2DM and in non-diabetic patients, independent of conventional cardiovascular risk factors and of the eGFR.

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Prediction of type 2 diabetes in angiographied coronary patients with the novel metabolic syndrome consensus definition: the importance of waist circumference

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Background: Recently, international societies including IDF, NHLBI, and AHA have put forth a novel consensus definition of the metabolic syndrome (MetS). Its power to predict the incidence of type 2 diabetes (T2DM) is unknown.

Materials and methods: We prospectively recorded the incidence of T2DM over 8 years in a population of 506 non-diabetic Caucasian patients undergoing coronary angiography for the evaluation of coronary artery disease.

Results: At baseline, 49·8% of our patients had the MetS according to the novel criteria when the lower IDF waist cut-offs were used and 39·7% when the NCEP-ATP-III waist cut-offs were applied. Prospectively, T2DM was newly diagnosed in 107 patients; the incidence rates of T2DM significantly

increased from subjects without the MetS over the intermediate group who had the MetS with the lower IDF waist cutoffs to patients who had the MetS also when the more selective NCEP- ATP-III waist cutoff values were applied (13·8%;15·7%;31·8%; P trend $< 0{\cdot}001$). After multivariable adjustment T2DM risk was significantly higher in patients with a MetS diagnosis based on the selective NCEP-ATPIII waist cutoffs than in the intermediate group [odds ratio 2·54 (1·12–5·73); $P = 0{\cdot}025$].

Conclusions: The 8-year incidence of diabetes in non-diabetic patients undergoing coronary angiography who meet the novel MetS criteria is very high, especially when the more selective NCEP ATP-III waist circumference cutoff values are applied.

Workshop 11: Stress and the skin

1101
The sebocyte-own corticotropin-releasing hormone system is an amplifier of inflammation

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Activation of the hypothalamic-pituitary-adrenal axis, which is the main adaptive response to chronic systemic stress, requires production of corticotropin-releasing hormone (CRH). CRH has also been detected in peripheral tissues, including the skin. Human sebaceous glands and cultured human SZ95 sebocytes express CRH, CRH-binding protein (CRH-BP) and CRH receptors (CRH-R) 1 and 2 at the mRNA and protein levels as we have shown by RT-PCR and immunohisto- and immunocytochemistry. CRH and urocortin, a peptide which shares a 45% homology with CRH, inhibit SZ95 sebocyte proliferation, which can be restored by α-helical-CRF, a CRH antagonist. CRH upregulates 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase mRNA levels and testosterone accordingly downregulates CRH-R1 and CRH-R2 mRNA expression in a negative feedback manner. Moreover, CRH enhances the basal synthesis, while antalarmin, a non-peptidic selective CRH-R1 antagonist, reduces the increased production of neutral lipids in SZ95 sebocytes. CRH also stimulates interleukin (IL)-6 and IL-8 release from SZ95 sebocytes but has no effect on IL-1α and IL-1β production or the IL1β-induced IL-8 release in these cells. Inflammation, such as acne, and proinflammatory signalling, such as UVB or the bacterial antigen MALP-2 amplify intracellular CRH levels, while dexamethasone inhibits the intracellular CRH synthesis in SZ95 sebocytes. No CRH release could be detected; CRH-BP expression was amplified in differentiating sebocytes of acne-involved skin. In conclusion, inflammatory signals can enhance intracellular CRH levels in human sebocytes leading to CRH-R1-associated lipogenesis and amplification of inflammation by secretion of proinflammatory cytokines. These events are sensitive to dexamethasone or specific CRH-R1 antagonists.

1102
Stress and psoriasis

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Psoriasis is a multifactorial inflammatory skin disease that affects 2–3% of the population in Europe. Immune regulation plays a central pathophysiological role in the development of psoriasis, an autoimmune condition with a complex genetic basis. Psoriasis is characterized histologically by increased proliferation of keratinocytes and by inflammatory leukocyte cell infiltration (mainly TH1 and Th17 cells) into the epidermis and the underlying dermis. Moreover, various environmental factors contribute to the manifestation of the disease, and psoriasis is often characterized by significant mental health issues. Psoriasis patients commonly report a significant decrease in their quality of life and high rates of depression, suicidal thoughts, increased perceived stress levels, and social stigmatization. Psychological or life stressors have been reported to precede the onset of psoriasis, as well as to precipitate flares of the disease. Such heightened levels of distress in psoriasis patients are likely to affect the disease via stress-responsive hormones released in the circulation or in the skin. Evidence is accumulating to support the existence of a hypothalamo-pituitary-adrenal axis equivalent within the skin, with a stress-triggered, localized secretion of corticotrophin-releasing hormone, adrenocorticotropic hormone, glucocorticoids, nerve growth factor, calcitonin gene-related peptide, and substance P. Elevated expression levels of these substances have been observed in lesional skin on the gene and protein level. Thus, the inflammatory disease psoriasis provides strong evidence for a bidirectional interaction between the brain and the skin inflammation.

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Expression of TNF superfamily members BAFF, APRIL, TWEAK, and their receptors in human normal and pathological skin

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Skin keratinocytes produce different cytokines, influencing the migration of inflammatory cells in the skin, as well as their

proliferation and differentiation. We examined the expression of the TNF superfamily members BAFF (TNFSF13B), APRIL (TNFSF13), their receptors BAFF-R (TNFRSF13C), TACI (TNFRSF13B) and BCMA (TNFRSF17), as well as the expression of TWEAK (TNFSF12) and its receptor Fn14 (TNFRSF12A) in normal and pathological human skin, in an attempt to reevaluate the role of skin as an immunological organ. Normal skin keratinocytes express APRIL, BAFF and their receptors (BCMA, TACI), in a distinct spatial and temporal pattern. In the epidermis APRIL and BCMA are expressed mainly in the basal and spinous layers, while BAFF and TACI are expressed in the suprabasal layers. On the contrary, BAFFR is not expressed in the epidermis. APRIL and BAFF induce through NFκB activation the expression of IL-6 and GMCSF, thus being potentially involved in skin inflammation. Interestingly, APRIL and BCMA expression is strongly enhanced in psoriatic skin lesions, while BAFF and TACI expression does not show a similar enhancement. Furthermore, TWEAK is selectively expressed in basal keratinocytes, while it is absent from the upper epidermal layers. Fn14 follows TWEAK's expression, being present mainly in basal keratinocytes, and in a lesser extent in spinous and granular keratinocytes. Finally, TWEAK has been found to induce keratinocyte apoptosis through activation of Fn14 through a caspase- and a cathepsin B independent pathway. Concluding, TNF superfamily members BAFF, APRIL and TWEAK seem to be implicated in regulation of skin immune processes and inflammation. The present study poses new questions concerning the skin as an immunological organ.

1104
A novel role of endogenous Corticotropin-releasing hormone (*Crh*) on cutaneous wound healing

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Hypothalamic CRH, a major mediator of the stress response, is involved in the inflammatory response by exerting indirect anti-inflammatory effects via stimulation of glucocorticoid release as well as potent direct proinflammatory effects in a plethora of tissues, including skin. The latter is further supported by that, CRH and its receptors are expressed in human and murine skin. In the process of wounded skin repair, fibroblasts from the wound edges migrate into the wound and proliferate in order to fill the site of the wound. This process is highly coordinated and mediated by locally released growth factors and cytokines which likely act in an autocrine/paracrine manner. IL-6 is a pro-inflammatory cytokine critically involved in cutaneous wound healing. We have previously shown that CRH regulates IL-6 expression during inflammation and that *Crh* deficient mice have accelerated wound healing *in vivo* and suppressed tissue IL-6 expression. Our recent results show that *Crh*-/- fibroblasts have significantly compromised IL-6 and TGF-β secretion compared to that of *Crh*+/+ mice. Treatment of cells with CRH does not affect the secretion of either IL-6 or TGF-β in both genotypes. Furthermore, *Crh*-/- fibroblasts have significantly higher basal proliferation rate compared to *Crh*+/+ fibroblasts. Treatment with CRH has no effect on the proliferation rate of cells of either genotype. No difference is observed in apoptosis between the two genotypes. Furthermore, the number of *Crh*-/- cells migrated into the wounded area is significantly higher than that of *Crh*+/+ cells in the *in vitro* wound assay. Treatment of cells of either genotype with CRH does not alter their migration rate. Finally, in order to examine the effect of CRH on human fibroblasts, experiments using the CRF receptor antagonists, antalarmin and a-helical CRF(9-41) were performed. These experiments reveal increased proliferation and migration rate and suppressed IL-6 secretion of CRF_1 antagonist-treated fibroblasts. In summary, our *in vitro* and *in vivo* findings on the role of CRH in cutaneous wound healing demonstrate accelerated repair in the *Crh*-/- mice, possibly due to the increased migration and proliferation rate of the *Crh*-/- dermal fibroblasts. Our findings support the direct effects of CRH in skin biology and provide interesting insights for the potential usefulness of the developing specific agonists and antagonists of the CRH family in dermal injury or other skin diseases.

1105
Therapeutic efficacy of antineoplastic drug Ukrain depends on biological properties of tumor

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Background: NSC-631570 (Ukrain) – a semisynthetic compound of thiophosphoric acid and alkaloids from the plant *Chelidonium majus* – has been successfully used for more than 20 years for the treatment of benign and malignant tumors. However, the treatment regimen is not individualized. The aim of our work was to carry out a comparative investigation of the therapeutic efficacy of NSC-631570 in the treatment of experimental tumors with different biological properties in mice.

Materials and methods: NSC-631570 was administered intravenously to mice, bearing high-immunogenic melanoma B16 (low- and high-metastasizing variants) and low-immunogenic Ehrlich's carcinoma, seven times every third day, starting from the second day after the transplantation of tumor cells. The effect of therapy on tumor growth was evaluated by the indices of tumor growth inhibition in experimental animals. Cell cycle distribution of cancer cells was determined by flow cytometry. The metabolic activity of phagocytes (oxidative metabolism, arginase activity) was also estimated.

Results: Therapy with NSC-631570 was significantly more effective in animals with high-immunogenic melanoma B16. Among mice bearing melanoma B16 the therapeutic efficacy of drug was more expressed in mice bearing high-metastasizing tumor variant. High therapeutic efficacy was accompanied by inflammatory activation of phagocytes.

Conclusion: These results suggest that to increase therapeutic efficacy of cancer treatment with NSC-631570 it is necessary to take into account biological properties of tumor. Functional state of phagocytes can be considered as a one of prognostic criteria.

Workshop 12: Lifestyles and health. Mediterranean perspectives

1201
Wine and alcohol: good and bad news

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Wine drinking in moderation with meals has been for millennia an important component of Mediterranean lifestyle and still today wine represents the most common alcoholic beverage traditionally consumed in this area. Most important evidences about wine/alcohol consumption will be reviewed here. Observational studies provide some evidence that moderate alcohol consumption is associated with reduced all cause and cardiovascular mortality as well as with lower rates of coronary heart disease. Drinking in moderation may also reduce the risk of heart failure, diabetes mellitus, ischemic stroke, peripheral vascular disease and dementia.

Moderate alcohol intake seems to induce cardiovascular benefits mainly through its effects on HDL cholesterol and other plasma lipids, thrombotic activity and insulin sensitivity; other possible mechanisms include reduction of oxidative stress, apoptosis and inflammation.

Some reports suggest that cardioprotective effects are stronger for wine than beer and other alcoholic beverages, while, in this respect, there is no clear evidence of a superiority of red on white wine.

Studies concerning benefits of alcohol intake are not devoid of pitfalls, due to confounding factors and the fact that alcohol use is not distributed randomly among individuals. To date, no long-term randomized trial of alcohol administration exist.

Although moderate alcohol consumption may improve health, no level of alcohol drinking can be reliably considered as safe in some people. Health benefits of moderate alcohol consumption must be balanced against possible deleterious effect of alcohol on liver disease, cancer and accidents. This net risk-benefit balance differs in various age groups and populations. For example, people under the age of 45 may experience more harm than benefit from alcohol use.

Current scientific evidence is against recommendation of starting moderate alcohol drinking in people who currently do not use alcohol. In the absence of contraindications, Italian INRAN guidelines advice no more than two to three drinks (one drink is equal to 12 g of alcohol) daily for men, one to two drink daily for women and 1 drink daily for subjects of both sexes over 65.

1202
Hepatocellular carcinoma: from genetics to life style and natural history

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Background: The Hepatocellular Carcinoma (HCC) has relationship with known risk factors (HBV and HCV infection, alcohol, aflatoxinB1) and emerging risk factors life style related: obesity, diabetes, smoking, pesticides, coffee. By means of the Epidemiology of Delivered Care, we have realized a study on the impact and the risk factors of HCC in a region of the South of Italy (Puglia).

Methods: Data came from the hospital discharge forms of all Puglia resident patients from 2002 to 2008. The following malignant tumor of the liver ICD-9 codes were studied: 155·0 (primitive, that include HCC); 155·1 (biliary primitive); 155·2 (not specified if primitive or secondary).

Results:

1. No. forms with diagnosis of malignant tumor of the liver: 25·188 with 8·965 (36%) incident cases.
2. No. forms with 155·0 code: 18·819 (constant trend by the years but a modest increase in the last 3 years).
3. General variables: M: 72%, F: 28% (ratio M/F 2·5); average length of stay: 8·5 days; mean age of patients 69·2.
4. Main associated diagnosis: cirrhosis, diabetes (whose association causes a slight increase both in length of stay – 9·1 days and in mean age of the patients – 70·6 years), other hepatic diseases.
5. Divisions of discharge were (%): Internal Medicine (33·6); General Surgery (21·7); Gastroenterology (20·5); Infectious Diseases (10·8).

Conclusions: The methodology of epidemiology of delivered care is crucial for the collection of data to be used for management and planning of clinical pathways for most of diseases.

1203
Between genetics and life styles conditioning: the case of genotyping of oxidised LDL receptor 1 gene polymorphism in patients with metabolic syndrome

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Background: The lectine-like oxidized low-density lipoprotein receptor-1 (LOX-1), encoded by the OLR1 gene, has been implicated in the pathogenesis of atherosclerosis. We therefore evaluated the genotyping of OLR1 gene in a preliminary sample of 55 patients with Metabolic Syndrome, a clinical condition characterized by a high cardiovascular risk.

Methods and patients: The genotyping of the LOX-1 was performed by PCR analysis of the IVS4-14 A>G OLR1 polymorphism embedded within the OLR1 Linkage Disequilibrium block. Patients were assessed for routine serum parameters, microalbuminuria, insulin resistance (HOMA), and oxidative stress (Thiobarbituric acid reactive substances, TBARs and Thioredoxin).

Results: The allele or genotype distribution of the OLR1 IVS4-14 A>G was not statistically different between MS and controls subjects. A positive association was found between IVS4-14 GG genotype, microalbuminuria and fasting glycemia as well as a higher frequency of type 2 diabetes, elevated microalbuminuria, fasting serum glucose and HOMA index in the same subjects. Thioredoxin values were higher in MS patients but did not differ in relation to OLR1 IVS4-14 A>G

genotype. The ratio TBARs/Cholesterol was higher in MS both in IVS4-14 GG and IVS4-14 AG.
Conclusion: IVS4-14 GG genotype seems to be related to glucose metabolism disturbance, elevated insulin level and lipid peroxidation in patients with MS.

1204
Exhaled VOCs pattern to detect colorectal cancer. A new potential screening method

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Introduction: Analysis of the volatile organic compounds (VOCs) linked to cancer is a new frontier in cancer screening, in fact tumor growth carries several metabolic changes leading to the production of specific compounds (alkanes, benzene derivative, etc) which can be detected in exhaled breath. Since some VOCs concentration can be enhanced and other decreased, a specific pattern of breath VOCs and not a single VOC could probably better identify each type of cancer.

Aim of the study is to identify a breath VOCs pattern able to screening colorectal cancer patients
Methods: Breath VOCs from six colorectal cancer patients and six healthy controls were collected using a custom breath sampling device, adsorbed on sorbent cartridges and analyzed in TD GS MS (thermal desorber gas chromatography mass spectrometry). Each breath sampling was duplicated.
Results: The mean and SD of detected VOCs show that at least six of them belonging to the groups of BMAC (Breath Methylated Alkane Contour) and Aldehyde compounds can be considered interesting to cluster and distinguish patients from controls by multidimensional analysis of data.

Differences of these significant VOCs evaluated on replicated sampling of each patient breaths were of the same order of magnitude of SDs for the six considered patients.
Conclusions: Breath VOCs analysis is a promising methodology with potential clinical application in colorectal cancer screening.

1205
Noninvasive characterization of patients with nonalcoholic liver steatosis in the primary care setting. The 'VARES' italian multicenter study

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Background: Non-alcoholic fatty liver disease (NAFLD) is an emerging health problem worldwide which can be seen as the hepatic manifestation of the metabolic syndrome and holds an increased cardiovascular risk. NAFLD encompasses liver abnormalities ranging from simple steatosis to inflammatory steatohepatitis (NASH), advanced fibrosis, and liver cirrhosis. Whereas liver ultrasonography can easily detect steatosis, liver biopsy with histology is the ultimate invasive diagnostic tool to quantify the grade of inflammation, fibrosis, and cirrhotic changes. Thus, NAFLD represents a challenge in family medicine, since physicians need noninvasive tools to identify those patients at risk of evolution and requiring consultation.
Aims and methods: To noninvasively assess subjects with clinical features of NAFLD in a national primary care setting using Fibromax® (Biopredictive, France), an algorhytm including serum parameters plus age, sex and BMI, and scoring grade of inflammation, fat infiltration and fibrosis (provided by Istituto Biochimico Italiano (IBI), Latina, Italy).
Results: We examined 259 consecutive subjects (mean age 51 ± 10 years, range 18–65; males 165; less than one drink per day) with ultrasonographic steatosis (mild 16·2%, moderate 69·9%, severe 13·9%). Subjects were normal weight (11·5%), overweight (42%), obese [46·5%, (4·2% severe)]. Diabetes, blood hypertension and hyperlipidemia were present in 24·7%, 40·9%, and 56·4% of the cases, respectively (prevalence of metabolic syndrome: 29·7%). Serum liver transaminases were elevated in 60·2% of subjects. Normal weight patients had normal transaminases in two/third of cases, none was diabetic but all of them were hyperlipidemic or were taking steatogenic drugs. A multivariate logistic regression including age > 50, BMI > 30, hypertransaminasemia, HOMA > 3 identified 12·3% of patients likely carrying inflammatory/fibrotic changes (NASH) at increased risk of evolution. By Fibromax®, 34 patients (13·1%) likely had advanced fibrosis while over 28% of those with moderate steatosis at US likely had severe steatosis with a score significantly related with ALT, BMI, waist circumference, serum triglycerides, and with the fatty liver index which identified 73·4% of patients as likely carrying a fatty liver. Fibrotest related only with ALT levels.
Conclusion: Searching for NAFLD is an essential step among dysmetabolic patients because of the implications with increased cardiovascular risk and potential hepatic implications. This also applies to several asymptomatic patients with chronic liver disease. Further studies are ongoing to see if Fibromax® is a promising tool in primary care to better select NAFLD patients needing consultation or targeted therapies.

1206
'Good & Bad' Lifestyle habits in a cohort of young Italian medical students in Southern Italy. The 'medstyle' survey

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Background: The prevalence of the metabolic syndrome (MS) is increasing worldwide at any age, putting the populations at risk of major health problems: diabetes mellitus, obesity, hypertension, cardiovascular diseases, and liver steatosis. Appropriate management of MS includes the 'step-one' approach: i.e. physical activity and (Mediterranean-like) diet. Little information is available, however, about lifestyle habits in young people before clear stigmata of MS appear.
Aim and methods: To assess most frequent lifestyle habits in an homogeneous cohort of young subjects living in southern Italy. Between September 2010 and January 2011, a cohort of 158 healthy medical students was chosen, as a representative sample of 2nd–4th year students at the University Medical School in Bari, Italy (average of 300 students/year during 6 years). The protocol included a brief physical examination (history, waist circumference with cut-off values for visceral adiposity by IDF and ATPIII, Body Mass Index – BMI), administration of a specific custom-designed questionnaire (MEDSTYLE) and use of Winfood® software (Medimatica, Teramo,

Italy) for assessing basal metabolic rate, physical activity, daily average diet, 'junk food' consumption, smoking and drinking habits.

Results: The study comprised 90 (57%) Females (age 21·6 ± 0·2 SE years, range 18–30, median 21, BMI 21·0 ± 0·3 kg m^{-2}) and 68 (43%) Males (age 21·5 ± 0·2 years, range 19–26, median 22 BMI 23·2 ± 0·3 kg m^{-2}). Waist circumference was smaller in F than M (vs. and 74·5 ± 0·9 cm vs. 87·9 ± 1·3 cm, respectively, $P < 0·000001$). Percent overweight and obese subjects tended to be lower in F than M (7% vs. 13% and 1% vs. 4%, respectively, $P = 0·059$). Prevalence of subjects with visceral adiposity was higher by IDF than ATPIII: 30% and 7·8% in F ($P = 0·0001$) and 22% and 10% in M. Overall, daily caloric intake was 1412 ± 25 kcal in F vs. 1833 ± 40 kcal in M ($P < 0·000001$) gained across 4·3 ± 0·1 meals, with 99% F and 96% M having regular breakfast. Prevalence of cigarette smoking (14·4% vs. 19·1%) and alcohol consumption (76·5% vs. 62·2%, $P = 0·056$) tended to be lower F than M. Number of weekly alcoholic drinks were 2·0 ± 0·3 in F and 2·2 ± 0·3 in M with alcohol intake significantly lower in F than M: 12·4 ± 2·5 g per week vs. 20·0 ± 2·6 g per week ($P = 0·038$). Also, consumption for spirits, wine, and beer was invariably smaller in F (32·2, 44·4%, and 47·8%) than M (41·2%, 54·4%, and 69·1%). Sedentary life (i.e. no consistent weekly physical activity) was similar between F and M (60·0% vs. 51·5%). In those performing physical activity, no. of weekly activities were 2·7 ± 0·2 for 88 ± 8 kcal per day in F and 2·5 ± 0·2 day or 113 ± 14 kcal per day in M (P = NS). Daily consumption of extravirgin olive oil was 23·3 ± 1·0 g per day while estimated sodium intake with pasta was 6·6 ± 0·3 g per day, without sex differences. Intake of solid-liquid junk (fast) food based on a frequency-size 7-items composite score, was greater in M than F (19·3 ± 0·8 vs. 16·8 ± 0·6, $P = 0·015$) and tended to correlate positively with abdominal girth in the whole group ($n = 158$, $r = +0·28$; $P = 0·052$). Although the frequency of legumes consumption was comparable, the score for size of legume intake was significantly smaller in F than M 1·6 ± 0·07 vs. 1·9 ± 0·09 ($P = 0·0028$). The same applied to milk consumption (score F 1·8 ± 0·1 vs. M 2·2 ± 0·1, $P = 0·032$).

Conclusions: This survey provides a consistent anthropometric and lifestyles database in an homogeneous cohort of young healthy medical students living in Apulia, Italy, a typical Mediterranean region. Initial clues to metabolic changes and 'bad' lifestyles are seen already at this stage including smoking, drinking, increased visceral fat-overweight-obesity, high intake of sodium salt, junk food, and sedentary life. Observational and prospective surveys are ongoing to depict the natural history of metabolic disorders at an early stage and to start better educational healthy programs.

1207
Promoting healthy lifestyle and moderate alcohol consumption in working class *heroes*

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Background: WHO estimates that 10% to 30% of industrial accidents may be due to alcohol consumption and a recent italian law establishes guidelines for the prevention of these accidents. In order to get insights into alcohol consumption styles and other lifestyles, we performed an extensive survey among almost 3000 people of different working class sectors.

Methods: The health survey questionnaire (Wallace, 1987) was used in combination with CAGE questionnaire (2530 cases, M/F:4·8; range 16–69 years) or the Alcohol Use Disorders Identification Test (AUDIT; Fiellin, 2000) (593 cases; M/F:2·1; range 17–69years).

Results: Distribution of BMI was: 25–29·9: 36·7%; ≥ 30:11·4%. Physical activity is done by 48% of males and only 36% of females; smoking: 47% of males, 38% of females; fruit consumption: > 95% in both sex; alcohol consumption: 76% of males, 47% of females; depression feeling: 2% of males, 4% of females; In the CAGE group, 2% of people (only males) gave three or four positive answers (hazardous or risk drinking, high risk of alcoholism) and 7·5% two positive answers (suspect of risk drinking). In the AUDIT group, 3·5% of people (only males) gave greater than six positive answers (hazardous or risk drinking). In both groups, a positive correlation was found between drinking and incidence of accidents at work.

Conclusions: Alcohol, overweight and smoking are primary health problems in working class and claim for intensive preventive actions.

1208
Angiotensin II type 1 receptor, but no type 2 receptor, interferes with the insulin-induced nitric oxide production in HUVECs

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Two subtypes of angiotensin II (ATII) receptors have been defined on the basis of their differential pharmacological and biochemical properties: ATII-types1 receptors (AT_1-R) and ATII-type2 receptors (AT_2-R). It has been hypothesized that part of protective effects on cardiovascular system of AT_1-R blockers are mediated by an ATII-mediated overstimulation of AT_2-R. We hypothesize that the inhibition of AT_1-R has a stronger impact than ATII-mediated overstimulation of AT_2-R on insulin induced nitric oxide (NO) production. Therefore, we studied in Human Umbilical Vein Endothelial Cells (HUVECs), the effect of the blockade of AT_1-R and AT_2-R on ATII mediated actions. We studied the phosphorylation state of IRS1 on Ser^{616} and Ser^{312} and on tyrosine after preincubation with PD123319, an inhibitor of AT_2-R, alone and in combination whit losartan, an inhibitor of AT_1-R. In addition we have measured the eNOS and Akt activation measured as phosphorylation on Ser^{1177} and Ser^{473} respectively. ATII induces IRS-1 phosphorylation at Ser^{312} and Ser^{616} by activation of JNK and ERK½, respectively. This results in an inhibition of the insulin-induced tyrosine phosphorylation of IRS1 and serine phosphorylation of Akt and eNOS. Treatment of HUVECs with AT_1-R inhibitor restored the insulin signalling leading to NO production, whereas AT_2-R inhibitor did not have effects on NO production in presence of ATII. Our results demonstrate that in presence of AT_1-R antagonism the AT_2-R blockage doesn't modify the effect obtained with the AT_1-R inhibition alone. Therefore, a possible positive role of an AT_2-R overstimulation in condition of AT_1-R antagonism seems to be irrelevant.

1209
Cytokine pattern, EPCs circulating levels and vascular stiffness in essential hypertension

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Endothelial progenitor cells (EPCs) activate and preserve the integrity of vascular angiogenesis, contributing to the prevention of atherosclerosis. Their number and functional capacity are reduced in patients with essential hypertension (EH). Endothelial dysfunction and arterial stiffness (AS) represents an early stage in the development of atherosclerosis and are valid indicators of subclinical organ damage. Therefore, we evaluated the association between AS parameters and the levels of circulating EPCs and inflammatory cytokines in patients with EH.

We enrolled 60 untreated hypertensive patients without cardiovascular complications or diabetes mellitus. The AS was measured by carotid-femoral pulse wave velocity (PWV), using a semiautomatic tonometer. The pool of circulating EPCs was determined by flow cytometric analysis of surface antigens: CD34, CD133 and VEGFR2 (KDR). The cytokine network was evaluated in plasma by the 'Multi-Analyte biochip array' system (Randox Laboratories) with the simultaneous determination of IL2, IL4, IL6, IL8, IL10, IL1α, IL1β, TNF, IFNγ, MCP-1, VEGF, EGF. The data showed that the PWV is inversely correlated with circulating levels of EPCs and directly correlated with the levels of TNFa, IL-6, and less with the levels of IL-1β. Finally, the circulating levels of EPCs are inversely correlated with IL-6, TNFa and IL-1β. The stepwise multiple regression analysis showed that levels of EPCs are the strongest independent predictor of PWV. Therefore, circulating levels of EPCs, as well as those of some inflammatory cytokines, are closely related to AS. In the presence of elevated circulating levels of inflammatory cytokines, especially of TNFa, low levels of EPCs are associated with an increase of AS, which promotes the onset and progression of atherosclerosis.

1210
Obesity and angiotensin type-1 receptors overexpression

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Obesity is an important risk factor for the development of multiple comorbidities and, above all, for the development of cardiovascular disease and type 2 diabetes. Obesity, especially the abdominal one, is associated with a pro-inflammatory condition. Adipokines may link obesity to its co-morbidities. In addition, renin-angiotensin-aldosterone system (RAAS) may represent an important link between obesity and hypertension. There is evidence that angiotensin II (Ang II) could have an effect on glucose metabolism interfering with the insulin signaling cascade that leads to a reduced glucose uptake. The main effects of Ang II, such as vasoconstrinction, aldosterone release, sodium and water reabsorption and blood pressure increase, are mediated by Ang II-type 1 receptors (AT1), even if their metabolic role remains unclear. In the present study we evaluated the differences existing between normotensive-normal weight (N-NW), hypertensive-normal weight (H-NW), normotensive-obese (N-O) and hypertensive-obese (H-O) subjects about the AT1 density (AT1d) on platelets, testing the hypothesis that the obesity is a condition in which the RAAS may be overactive. We enrolled 24 subjects, in absence of any pharmacological treatment, divided into four groups: N-NW, H-NW, N-O and H-O patients. BMI was calculated and the waist circumference (WC) was utilized as correlate of the distribution of visceral adipose tissue. Platelets were isolated and purified from venous blood samples and a binding assay with radiolabelled Ang II was performed to establish the amount of AT1 on platelets. AT1d progressively increases from N-NW group to H-NW, N-O and H-O. AT1d is significatively related with WC, BMI, insulin levels and estimated glomerular filtration rate. In a regression stepwise model, the only independent predictor of AT1d is the WC, that influences over 40% of its variation. Thus we have observed that N-O patients show the greatest AT1d than the control group and the H-NM one, while the hypertensive condition, associated with obesity, increases only the 15% of AT1d. This evidence leads to suppose that the activation of RAAS, more evident in the obese patients, is oriented to modify the insulin-sensitivity condition of these subjects, confirming the hypothesis that obesity is the major risk factor in the development of diabetes and cardiovascular events.

1211
Uric acid promotes endothelial dysfunction: a new molecular model of insulin resistance

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Hyperuricemia is associated with hypertension, type-2 diabetes, metabolic syndrome, vascular and renal disease and cardiovascular (CV) events. Uric acid (UA) is a marker of (CV) risk and it remains controversial if it is has a causative role. A reduction in nitric oxide (NO) bioavailability leading to a reduced endothelium-dependent vasodilation has been identified as a key pathogenic event anticipating the development of hypertension, type 2 diabetes and cardiovascular disease. The reduced NO bioavailability has been attributed to increased production of reactive oxygen species (ROS) that either directly react with NO or uncouple its substrate enzyme. It is well established that insulin signaling pathway stimulates NO production by activating the PI3K/Akt/eNOS axis. In this study we evaluated, in human umbilical vein endothelial cells (HUVECs), whether UA negatively modulates insulin signaling pathway leading to NO production. HUVECs were cultured in ECM medium and stimulated with insulin and UA at the concentrations of 10^{-7} M and 10 mg dL^{-1}, respectively. Activity of the signaling proteins was detected by Western Blot analysis, using antiphosphospecific antibodies (anti-eNOS, p-eNOS Ser^{1177}, Akt and p-Akt Ser^{473}). The amount of NOS activity produced by HUVECs was assayed using a NOS Detect Assay Kit, that measures the ability of NOS to convert L–^{14}C-arginine to L–^{14}C-citrulline. UA inhibits the insulin-induced Ser^{1177}-eNOS phosphorylation through an Akt inhibition; from this event results an impairment into insulin-pathway leading to NO production. According with these findings it is possible to hypothesize that inhibition of Ser^{1177}-eNOS phosphorylation induced by UA is associated with a significant reduction in the NO production. In HUVECs UA negatively regulates the insulin actions reducing the NO production through an impairment of Akt/eNOS pathway.

Our data suggest that UA exerts a negative role in the insulin signaling; the effects of hyperuricemia could be relevant for the onset and the worsening of insulin resistance in course of diabetes, hypertension and obesity.

1212
Analysis of molecular mechanisms and anti-tumoral effects of zoledronic acid in breast cancer cells

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Background: Zoledronic acid (ZOL) is the most potent bisphosphonate, clinically available for the treatment of patients with osteoporosis and bone metastases, because these compounds bind strongly to bone mineral and are powerful inhibitors of bone resorption.

In preclinical studies, strong anti-cancer activities against breast cancer, prostate cancer, and leukemia have been reported, but there is increasing interest in their potential role in preventing and treating cancer-induced bone loss and their possible direct anti-tumor effects.

However, recent data suggest that bisphosphonate use in breast cancer may provide more than just supportive care and modify the course of the disease, though is unclear the possible molecular mechanism of action.

Materials and methods: Since ZOL has the tendency to accumulate in bone, we investigated the effect of ZOL on MCF7 breast cancer cells, to identify, describe and summarize evidences regarding the molecular basis of actions of bisphosphonates and of their possible anti-tumor effects.

Results: We have observed for the first time gene expression profile by Microarrays analysis in MCF7 treated whit ZOL and specific transcripts confirmed by Real Time RT-PCR. Relative proteins, that correspond to functional products were assayed by Western Blot analysis and migration capacity of MCF7 cells, relayed by ZOL, were observed on Matrigel.

Conclusions: Here, we have provided evidence that BP pretreatment of breast carcinoma cells inhibited tumor cell proliferation, cellular adhesion, invasion and migration and that inhibited angiogenesis in breast cancer cells.

1213
Assessing life style contribution to cardiometabolic risk: an intranet based approach

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Objectives: In the present investigation we tested the feasibility of using a private website, directed to the workers of a major multinational company, to assess the health profile and life style and work habits using an *ad hoc* self administered questionnaire.

Methods: Anonymous multiple choice web based questionnaire administered to 945 subjects (683 completed the task) as part of an ongoing health promotion program in a multinational company. Data were explored with nonlinear principal component analysis in order to focus on life style and cardiometabolic indices. Combining qualitative and quantitative data we explored clustering of risk factors with life style components.

Results: Stress perception dimensions were directly correlated to heart rate, while activity was inversely correlated to biochemical indicators of cardiometabolic risk, (Diastolic) Arterial pressure and heart rate. Alcohol consumption predicted systolic and diastolic arterial pressure. Computed clusters reinforced the inverse profile of stress and activity, and suggested that these domains can partly predict absenteeism.

Conclusions: The present approach appears feasible and underscores a significant relationship between lifestyle (exercise and stress), indicators of quality of work (reported absenteeism) and of cardiovascular risk, as inferred from reports of metabolic and hemodynamic data (in particular arterial pressure).

1214
The olive oil chemistry in Apulia, a typical mediterranean region

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Background: World-wide production of olive oil during the last 20 years has increased by almost 70%. The authenticity of olive oil is an important issue from a commercial and health point of view. In recent years the interest in extra virgin olive oils has grown; due to its recognized nutritional value, extra virgin olive oil is considered as a fundamental component of the Mediterranean diet. However, olive oil is a complex mixture consisting of two main groups of substances: saponifiables and unsaponifiables compounds, and the exact knowledge of its composition is of therefore of key importance.

Materials and methods: Thirty olive oil samples were collected from various producers working in different areas of the Apulia region and the harvest was analyzed at the Oliveti d'Italia laboratory. All samples considered in this study were virgin olive oils according to the official analytical methods and limits (EC, 1991). Determinations of free acidity, peroxide value, conjugate diens and triens (K232, K270), fatty acid concentrations were carried out according to official analytical methods including UV, gas chromatography, and titration.

Results: The concentrations of analyzed compounds were always within the normal cutoff values. In particular, mean value for acidity was 0·2% (cutoff 0·3%), for peroxide 7 eq/O_2 (cutoff 22), for K232 1·8λ (cutoff 222), for K270 0·13λ (cutoff 0·22). For fatty acids, results showed that oleic acid concentration was max 78% (cutoff 83%), linoleic acid concentration was 7% (cutoff 21%) and linolenic acid was 0·5% (cutoff 1·0%). Overall, results confirmed the authenticity of examined samples of olive oil.

Conclusions: Our database allows the exact classification of factors responsible for qualitative variability of Apulian olive oils. Concentrations of fatty acids such as oleic, linoleic, and linolenic acid show a narrow variability in olive oils from different Italian geographical areas; this feature is used to discriminate Italian olive oils from foreign olive oils, since the variability of fatty acid concentrations is greater if olive trees grow in extremely different pedoclimatic conditions. Further measurements are ongoing to compare oil samples from Apulia with olive oils from foreign countries.

1215
Mediterranean lifestyle and thalassotherapy

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In recent decades nutrition has gained great importance for the state of human health, aging and the prevention and treatment of a variety of diseases. Lifestyles (e.g. diet and physical activity) represent one of the most important modifiable risk factors for the development cardiovascular disease, and metabolic disturbances. The Mediterranean diet, now a recognized UNESCO world heritage, is an example of proper and balanced nutrition, and is associated with a reduced incidence of cardiovascular morbidity (1) and overall mortality for cancer and neuro-degenerative diseases (2). Exercise is associated with reduction in blood pressure and blood levels of inflammatory markers, improved insulin resistance and lipid profile overall (mainly causing a significant increase in HDL cholesterol) (3). The Centre for Research and Training on Thalassotherapy of Magna Graecia was recently developed in order to promote health programs focusing on both diet and physical activity.

Methods: A multidisciplinary staff was organized, and composed of MDs, biologists, trained nurses and specialized workers (i.e. physical trainees, chefs, psychologists etc.). Upon arrival, residents undergo an appropriate educational programme, a general medical screening to rule out major and debilitating organic diseases, careful identification of risk factors for metabolic syndrome, abdominal ultrasound for assessment of liver steatosis and gallstone disease (two additional components of the metabolic syndrome). The first 3 days subjects were instructed and started a balanced mediterranean diet with appropriate intake of fats (prevalence of mono- poly-saturated fats, rather than unsaturated fats of animal origin), and an adequate intake of anti-oxidants (at least five courses a day between fruit and green vegetables), no alcoholic beverages. Calculation of daily kcal intake was based on body size and metabolic rate, according to the Harris-Benedict equation. In all subjects, the programme included a moderate aerobic physical activity (equivalent to a daily consumption of 200 kcal) including walking, cyclette, and treadmill.

Results: An initial group of 90 subjects were asked to join the programme at no additional costs. Acceptance rate was 55·6% equivalent to 50 subjects (25 couples, mean age 45 years). This cohort was checked again after 3 months. The 3-day programme was completed by all couples (100%), while the 3-month programme was completed by 15 couples (60%). In this case, weight maintainance was observed in all lean subjects; among the nine overweight-obese subjects, a mean reduction of 5% body weight was observed in seven subjects. Major reason to stop the 3-month programme was the need for continuous physical exercise, and difficulty to understand and use a 'simple' mediterranean diet at home.

Conclusions: With the increasing prevalence and incidence of the metabolic syndrome, and increased cardiovascular risks and mortality in the general population worldwide, a better knowledge of health benefits related with lifestyle changes represents an urgent and unmet need. Our experience suggests that educational programs should be easy to follow, poorly expensive, and linked to well-trained medical and professional staff. The Mediterranean diet and constant daily physical exercise are the mainstay of first-step treatment in populations at risks of dysmetabolic conditions.

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1216
Persistent secondary hyperparathyroidism after renal transplantation: effects of therapy with cholecalciferol

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Hypovitaminosis D is a frequent condition in kidney-transplanted patients. Vitamin D insufficiency/deficiency is an important risk factor for the persistence and severity of secondary hyperparathyroidism (SHPT) and thus for skeletal morbidity. The aim of this study was to evaluate the effect of a therapy with vitamin D on PTH levels in kidney-transplanted patients.

We studied 50 patients (38 males and 11 females), who had undergone kidney transplantation 0–10 year before, aged 30–70 years (M ± DS 52.62 ± 10.78), with serum creatinine ≤ 227 μmol L^{-1}. After four weeks of pharmacological washout, all patients underwent an evaluation of biochemical parameters of bone metabolism: 25-OH vitamin D, serum calcium, phosphate, PTH and creatinine (t_0). The same parameters were re-evaluated after 4 (t_1) and 8 (t_2) months of therapy with vitamin D (cholecalciferol, 30 drops once a week equal to 7500 IU), respectively. At t_0, all patients showed 25-OH vitamin D levels lower than 75 nmol L^{-1} (M ± DS 32.02 ± 14.95 nmol L^{-1} = insufficiency). After 4 months of therapy, vitamin D levels increased to 62.98 ± 13.71, not yet up the normal value. After 8 months of therapy, there was a further increase in vitamin D levels. (M ± DS 70.28 ± 20.20). At the end of the study period, only in 18% of patients 25-OH vitamin D was normal (> 75 nmol L^{-1}). As far as PTH levels were concerned, we observed a slight decrease of its value. In conclusion, it is possible that kidney-transplated patients may need higher values of vitamin D with respect to those given in the present study, for achieving full vitamin D repletion and thus a more important decrease in PTH levels.

1217
Hypovitaminosis D in a acute ward of internal medicine

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Hypovitaminosis D is a frequent condition in the general Italian population. The aim of this study was to evaluate the prevalence and the clinical meaning of hypovitaminosis D, in a population of hospitalized subjects in a Division of Internal Medicine.

We studied 164 female patients, aged 22-97 years (M ± DS: 78 ± 12), with Barthel Index at the discharge from hospital of 70 ± 32 (0–100). Nine of them were excluded from statistical analysis because hypercalcemic, six with primary hyperpar-

athyroidism, one with iatrogenic hypercalcemia and the other one with malignant hypercalcemia. The more representative discharge diagnoses were congestive heart failure, coronary heart disease, other cardiovascular diseases, gastrointestinal and respiratory diseases, neoplasia and cerebrovascular diseases. Eighty-two percent of the patients suffered from different comorbidities. The mean values of serum 25-OH-D were 30.6 ± 26.1 nmol L^{-1}. Nine patients (6%) were vitamin D replete, 48 patients (31%) were vitamin D insufficient and 98 patients (63%) were vitamin D deficient. Only 13% of the patients were taking oral calcium and vitaminD supplements. The mean value of PTH was 124.8 ± 113.3 ng L^{-1}. Bone AP was increased in 27 patients (17%).

25-OH-D values didn't change with age, educational level, season, sun exposure and dairy calcium intake with diet, whereas they were positively correlated with calcium-vitamin D supplements intake or only vitamin D supplements. Moreover, these values were not different according to the type of disease which led to hospitalization, neither with comorbidities. The main predictor of serum PTH levels was age.

These data confirm the high prevalence of hypovitaminosis D in hospitalized patients. The oral calcium and vitamin D supplementation in these patients could represent an effective preventive strategy.

Workshop 13: Molecular aspects of renal diseases

1301
Phenotypic changes of podocytes in diabetic renal disease

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Background: Podocytes undergo structural and functional changes in diabetic nephropathy, linked with progressive loss of normal kidney function.
Materials and methods: Human podocytes (TSV40-immortalized) were cultured in the presence of 5- (normal) and 25 mmol L^{-1}- (high) glucose for prolonged time periods. Their morphology and the expression of proteins linked to the filtering function of podocytes, which form a pivotal component of the renal permselective barrier, was examined.
Results: Sustained presence of high glucose resulted in progressive loss of the surface sialoprotein podocalyxin, which participates and maintains foot processes. These became effaced in high glucose. WT1 which has been presumed to control the expression of podocalyxin and nephrinappeared unaffected in western analysis, but WT1 isoforms in the range of 36–38kDa were severely reduced. The binding on WT1 to the podocalyxin promoter was also reduced by 60% with ChIP analysis. Moreover, important components of the slit diaphragms, including nephrin and ZO–1 were also down-regulated, as was CD10, an epithelial marker. Restoration of glucose to normal levels could only restore the expression of ZO-1. The expression of the other examined components could be rescued only if some levels of these proteins remained expressed, up to 8–12 weeks of culture in 25 mmol L^{-1} glucose, or in the presence of amino guanidine, an inhibitor of glucose-induced adducts (AGEs).
Conclusions: The sustained presence of high glucose and ensuing AGEs progressively resulted in irreversible loss of most differentiated markers of cultured podocytes, and also their specialized morphology possibly indicating a process of de-differentiation which was mediated by impaired interactions of WT1 and the podocalyxin promoter, at least in part. Preventing the formation of AGEs may contribute to maintain normal structure and function of podocytes.

1302
Proteomics in obstructive nephropathy

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Background: Renal fibrosis is the hallmark of many renal diseases leading to renal dysfunction.
Materials and methods: Novel macromolecules involved in renal fibrosis were explored using proteomic analysis in the well established model of unilateral ureteric obstruction (UUO), at early (2 days) and late (8 days) time intervals. From the lists of macromolecules exhibiting differential expression, we have focused on two of them, calreticulin and transgelin.
Results: Calreticulin is an endoplasmic reticulum resident protein. Proteomic data were further confirmed by biochemical and morphological techniques. Upregulation of calreticulin was observed in tubular epithelial cells. Therefore, we performed overexpression of calreticulin in HK-2 cells, a cultured proximal tubule epithelium cell line. We observed a switch to a more mesenchymal phenotype, an increase in cell motility, an increase in apoptosis and changes in the secretory profile. Many of these changes mimic the ''fibrotic'' phenotype of resident renal cells.

Transgelin is a cytoskeletal, actin-binding protein. Proteomic data on transgelin were further confirmed as well by biochemical and morphological techniques. Upregulation of transgelin was observed in interstitial fibroblast-like cells, especially distributed

around glomeruli and to a lesser extent around tubules. These cells were not part of the immune system. Many of them (but not all) were positive for aSMA, a classical marker for activated fibroblasts. Examination of renal biopsies from patients suffering from various renal diseases suggested that transgelin is highly expressed (more so than aSMA) also in the glomerulus, in cases of IgA nephritis and Focal Segmental Glomerulosclerosis.

Conclusion: The above studies suggest that both calreticulin and transgelin are important molecules playing a role in renal fibrosis and their use in future diagnostic studies should be considered.

1303
Notch3 is essential for the regulation of the renal vascular tone

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The Notch3 receptor is mainly expressed in vascular smooth muscle cells and participates in the development and maturation of vessels. Mutations of Notch3 in humans are associated with defective regulation of cerebral blood flow. The objective of the present study was to investigate the role of Notch3 in the regulation of renal hemodynamics. Experiments were performed in mice lacking expression of the Notch3 ($Notch3^{-/-}$). Arterial blood pressure and renal blood flow (RBF) were similar in $Notch3^{-/-}$ and wild type littermates under control conditions. Next, we measured transient changes of RBF and renal vascular resistance (RVR) to bolus iv injections of vasoconstrictors (norepinephrine, angiotensin II), or vasodilators (bradykinin, prostacyclin). Wild type mice increased RVR and decreased RBF in a dose-dependent manner following injections of norepinephrine and angiotensin II. In sharp contrast, RVR of $Notch3^{-/-}$mice varied little after boluses of norepinephrine and angiotensin II. Inversely, bradykinin and prostacyclin relaxed renal vasculature and transiently decreased RVR in wild type mice. However, both vasodilators had a negligible effect on RVR of $Notch3^{-/-}$ mice. In subsequent ex vivo experiments, afferent arterioles freshly isolated from $Notch3^{-/-}$ mice showed a deficient contractile response to angiotensin II. These vessels displayed a significantly decreased thickness of vascular wall compared to wild type.

These data show that Notch3 is necessary for the control of renal myogenic tone. A deficiency in the expression of Notch3 could have important physiopathological consequences in the adaptation of the renal function and structure to chronic increase of blood pressure.

1304
Periostin is an early marker of the progression of renal disease

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Introduction: Chronic Kidney Disease (CKD) is characterized by progressive decrease in renal function related to a progressive accumulation of fibrosis. Recent studies have shown that POSTN is a major signal of cardiac fibrosis. The aim of our study is to investigate the implication of periostin (POSTN) in CKD progression.

Materials & methods: We studied several models of nephropathy affecting different renal compartments: two models of hypertension-associated renal disease, transgenic mice overexpressing renin (RenTg mice) and Angiotensin II-induced renal fibrosis in rats, a model of glomerulonephritis (anti-GBM) and a model of tubulointerstitial disease after unilateral ureteral obstruction (UUO).

Results: In RenTg, anti-GBM and UUO mice, POSTN mRNA expression was highly increased in the early phase of progression of CKD. At the same time, the expression of other genes, well-known for their pro-fibrotic action, was also increased (TGF-β, collagen III, Twist). In Ang II rats, the increase of expression of POSTN occurred later and was correlated to the degree of the advancement of the disease. Interestingly, POSTN expression was localized around hypertrophic vascular wall and altered tubules.

In subsequent studies we performed UUO in POSTN knock out/β-galactosidase knock-in transgenic mice. X-gal staining was dramatically increased following UUO; the staining appeared to be mainly in the peritubular areas.

Conclusion: These results suggest that the increase of POSTN might be an initial and important event of CKD. Further studies are necessary in order to characterize the pathways involved in this increase and the therapeutic potential of drugs targeting POSTN in CKD progression.

1305
Shock wave therapy lessens renal fibrosis lesions induced by hypertension

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Introduction: Neoangiogenesis can be involved in renal repair. Extracorporeal low-energy shock wave therapy (SWT) is a new effective, noninvasive proangiogenic therapy which reduces ischemia in ischemic heart and limbs diseases.

Objectives: The objective of our study was to investigate whether SWT could lessen renal lesion induced by L-NAME administration, a NOS inhibitor in hypertensive rats.

Methods: To this end, 10 rats received L-NAME and salt for 4–5 weeks, whereas the control group consisted of eight rats received salt. When proteinuria over creatininuria exceeded 1000 g mol^{-1}, reflecting thus an important degree of renal injury, SWT was performed in the left kidney of five rats three times per week for 5 weeks under brief anesthesia. The five other rats received only anesthesia during the same period without SWT. In the end of the experimental period, we evaluated the effects of SWT on SBP, proteinuria, renal function, renal hemodynamics, renal structure, inflammation, cell proliferation and synthesis of proangiogenic factors.

Results: SWT tends to reduce SBP ($P = 0.10$) and renal dysfunction. SWT lessened glomerular ($P < 0.001$), vascular ($P < 0.001$) and tubulo-interstitial ($P < 0.001$) lesions induced by L-NAME. SWT reduced resistive index ($P < 0.05$) and restored blood flow velocity (BFV) of interlobular renal arteries ($P < 0.05$) suggesting a local vasodilatation. SWT reduced the

infiltration by macrophages ($P < 0.01$) and by lymphocytes ($P < 0.01$) and decreased the expresion of MCP-1 ($P < 0.01$), CD3 ($P < 0.01$) and cell proliferation ($P < 0.01$). Interestingly, renal endothelin-1 expression was reduced by SWT ($P < 0.05$), but VEGF and its receptor were unchanged.

Conclusion: SWT reduces chronic renal lesions induced by L-NAME in rats, especially the glomerular and vascular lesions by blunting inflammation and cell proliferation and producing local vasodilation. SWT could be a novel treatment to favour renal repair.