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Abstract

The expression of *Azgp1* gene, an adipokine involved in the mobilization of body reserves, was observed in mammary gland of ruminants. Its regulation by different dietary conditions suggests a potential role in the mechanisms controlling the composition of milk fat. The aim of this study was to evaluate the role of *Azgp1* during lactation. *Azgp1*$^{-/-}$ mice were compared to wild-type to determine its effects on milk fatty acid composition and offspring growth. To determine its effects on mammary gland, adipose tissue and liver gene expression, gene expression was analyzed using RT-qPCR *via* TLDA analyses. The body weight of *Azgp1*$^{-/-}$ mothers was slightly higher after parturition and at 10 days of lactation compared to the wild type. The milk polyunsaturated fatty acid content was increased in *Azgp1*$^{-/-}$ mice. Among the 40 genes studied, *Azgp1*$^{-/-}$ modified the expression of 9, 10 and 3 genes in mammary gland, adipose tissue and liver, respectively. These genes, involved in fatty acid synthesis, transport and triglyceride synthesis, were downregulated in *Azgp1*$^{-/-}$ mice showing a particularity during lactation. Changes in mammary gland gene expression may explain the modifications observed in milk fatty acid composition. This study supports a role of *Azgp1* on lipid metabolism, in particular in mammary gland, during lactation function.

Keywords: *Azgp1*$^{-/-}$, lactation, gene expression, lipid metabolism, mammary gland, adipose tissue, liver, mice

1. Introduction

The alpha-2-glycoprotein 1, zinc-binding 1 (ZAG encoded by *AZGP1* gene), is a soluble and secreted protein classified as an adipokine. It has been detected in various organs, including adipose tissue, liver, kidney, breast, lung and heart in rodents and human [1-4]. Adipose tissue and liver are the two major sites of *Azgp1* expression in rodents [3, 5]. ZAG plays a crucial role in the regulation of body weight, and *AZGP1* mRNA expression is negatively correlated with body mass index and body fat mass in humans [6-9] and rodents [10]. In *ob/ob* mice, the injection of human recombinant ZAG reduces body weight and body fat content without modification of feed intake [11]. ZAG is involved in lipid mobilization [12, 13] by stimulating lipolysis in adipocytes, both *in vitro* and *in vivo*. In addition to lipid mobilization, ZAG may also regulate the metabolism of excess fatty acids (FA) liberated from adipose tissue in periods of enhanced lipolysis [6]. In humans, subcutaneous adipose tissue *AZGP1* mRNA is correlated with adiposity, adipose tissue insulin sensitivity and expression of *GLUT4* (the facilitated glucose transporter, also known as *SLC2A4*), *PPARGC1A* (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha, a transcriptional coactivator that regulates the genes involved in energy metabolism), *IRS1* (Insulin Receptor Substrate 1) and *ADIPOQ* (adiponectin, an adipokine involved in the control of fat metabolism and insulin sensitivity) [14]. *In vitro* studies reported that *AZGP1*-knocked down hepatocytes increased the expression of genes encoding lipogenic enzymes [*FASN* (FA synthase), *ACACA* (acetyl-CoA carboxylase-1), *ME* (Malic Enzyme), *PRKAA2* (Protein Kinase AMP-Activated Catalytic Subunit Alpha 2) and *PRKAB* (Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 1)], and decreased expression of gene encoding ß-oxidation enzymes [*ACC2* (acetyl-CoA carboxylase beta) and *CPT1B* (carnitine palmitoyltransferase 1B)] [15, 16].

Previous nutrigenomic studies revealed the expression of *AZGP1* gene in the mammary gland (MG) of ruminants and demonstrated its regulation by nutrition [17, 18]. Indeed, 48-h food

deprivation of lactating goats decreased *AZGP1* gene expression in the MG. In addition, *AZGP1* expression in MG increased with extruded linseed and fish oil supplemented diet comparatively to extruded linseed alone [18]. These two nutritional studies showed modifications in milk FA composition suggesting a potential link between *AZGP1* gene expression and milk FA composition. Although the role of ZAG in obesity was well characterized, literature on the role of *AZGP1* on lipid metabolism in the MG during lactation and its influence on milk composition is scarce.

The objective was to study the effects of *Azgp1*$^{-/-}$ on MG, adipose tissue and liver gene expression and milk FA composition in lactating mice. We found that *Azgp1*$^{-/-}$ downregulated expression of genes involved in lipid metabolism, in adipose tissue and MG. Altered MG lipid metabolism could explain in part the modifications of milk FA composition observed in this study.

2. Materials and methods

2.1. Generation of the Azgp1$^{-/-}$ *mice.*

Azgp1$^{-/-}$ mice were generated using embryonic stem (ES) cells provided by the International Knockout Mouse Consortium. *Azgp1* gene (on chromosome 5) were replaced by a bacterial artificial chromosome (BAC) based vector obtained from BMQ BAC library (Mouse Micer vector set 97E22) containing a neomycin and a Lac Z reporter cassette (http://www.komp.org/geneinfo.php?MGI_Number=103163; Supplementary Fig. 1). The mutated ES cells were derived from VGB6 ES cells (C57BL/6NTac background). After expansion, three different mutated clones were injected into donor blastocysts and transplanted into pseudopregnant females at the “SFR Biosciences – AniRA - PBES" (“Plateau de Biologie Expérimentale de la Souris”, Lyon, France). Chimeric male offspring were selected and mated with the C57BL/6J strain to generate F1 offspring at UMR1019 Human Nutrition (Theix,

France). After phenotyping, only heterozygous black mice were kept among the offspring of the first generation (F1), whereas chimeras with a high percentage of agouti coat color were excluded. Mice were genotyped at each generation by PCR using primers described in Table 1. Mice were intercrossed at approximately 49 days of age to obtain homozygous *Azgp1*$^{-/-}$ and *Azgp1*$^{+/+}$ genotypes. After genotyping the offspring of the second generation (F2), only homozygous mice were again intercrossed (female A*zgp1*$^{-/-}$ × male *Azgp1*$^{-/-}$, and female *Azgp1*$^{+/+}$ × male *Azgp1*$^{+/+}$) in a total of 20 crossings for each genotype. After genotyping, the offspring of the third generation (F3) were again mated. The analyses were performed after two new mattings to produce F4 and F5 homozygous mice. The scheme of the establishment of the *Azgp1*$^{-/-}$ mouse line is presented in Supplementary Fig. 2. Mice were housed at the platform of animal experimentation of UMR1019 Human Nutrition (Theix, France, agreement number D6334515). Animals were maintained in a light/dark cycle of 12 h, under a controlled room at a temperature of 22°C and humidity of 45–55%, and fed a conventional diet *ad libitum* (consisting of 21.4% crude protein, 5.1% lipid, 3.9 % fiber, Scientific Animal Food & Engineering, 89290 Augy, France, Ref diet, A03). Experimental procedures and animal handling complied with the guidelines for animal research of the French Ministry of Agriculture and were approved by the Auvergne regional ethics committee for animal experimentation (agreement number CE 84–12).

Table 1
Primer sequences used for PCR-based genotyping of the *Azgp1*$^{-/-}$ mouse line.

Target	Forward sequence	Reverse sequence
Azgp1	TGGCTCGGTTGAGAGGATG	ACTTGGTTTATGTGGTGCTTAGG
LacZ	GGTAAACTGGCTCGGATTAGGG	TTGACTGTAGCGGCTGATGTTG

2.2. Litter growth, mother body weight and body composition

The effects of *Azgp1*$^{-/-}$ on litter growth, and interactions between nursing mother (20 females *Azgp1*$^{+/+}$ and 20 females *Azgp1*$^{-/-}$, aged 12-13 weeks) and litter genotype were studied by cross-feeding. All F4 pups were separated from their respective mothers and swapped within 24

h after birth, in a 2 x 2 factorial arrangement of treatments. This litter swap allowed to have 9 mothers $^{+/+}$ with pups $^{+/+}$, 13 mothers $^{+/+}$ with pups $^{-/-}$, 13 mothers $^{-/-}$ with pups $^{+/+}$, 8 mothers $^{-/-}$ with pups $^{-/-}$, with an adjustment of 4 pups per litter and foster mother. The litter was weighed daily from d0 until d20 postpartum. The weights of lactating mice were measured just after parturition and before litter swaps for F4 generation, and at d10 for F5 generation. The body composition of each lactating mouse at F5 generation was determined at d10 using the EchoMRI™ (Zinsser Analytic GmbH, Germany) method that estimates body fat mass and lean mass in live animals.

2.3. Milk sampling and analyses

At mid-lactation (d9), 20 *Azgp1*$^{+/+}$ and 20 *Azgp1*$^{-/-}$ mothers of F5 generation were separated from the litter for 2 hours before collection of milk samples, injected with 0.3 U synthetic oxytocin (CEVA Sante Animale, Libourne, France) and then anesthetized by intraperitoneal injection (0.01 mL/g of body weight) of a solution containing 1 mL Imalgène 1000 (Merial, Lyon, France), 0.6 mL Rompun (Bayer Pharma, Puteaux, France) in a final volume of 10 mL water. The MG was then massaged slightly to induce milk secretion into a hematocrit tube. Fat content in fresh milk was determined by the creamatocrit method [19]. Approximately 75 µl of whole milk was drawn into a standard glass capillary tube (75 mm in length, 1.5 mm and 1.2 mm in outside and inside diameters, respectively), centrifuged at 12,000 rpm for 12 min, and fat content was calculated by the following formula: $Y = [(X - 0.59)/0.146] \times 0.1$, where Y = fat content (g/100 ml) and X = percentage of cream length total sample in the tube (creamatocrit percentage). Fat samples obtained by capillary tube centrifugation were analyzed for FA composition after a one-step extraction and methylation. The FA Methyl Ester (FAME) profile was determined using a Trace-GC 2000 Series gas chromatograph and 0.6 µL of sample and a split ratio of 1:30, as previously described for FAME analysis of milk [20].

Chromatographic peaks were routinely identified by retention-time comparisons with commercial authentic standards containing mixtures of FAME [20].

2.4. Blood and tissue sampling

For plasma and tissue collections, the animals, in the fed state, were anesthetized prior to euthanasia by cervical dislocation. Anesthesia was induced by placing each mouse in an inhalation chamber containing 4% isoflurane regulated with a calibrated vaporizer.

After anesthesia, blood samples were immediately drawn from the periorbital sinus of eyes at d10 into evacuated tubes containing EDTA (1.95 mg/mL; Terumo Europe NV, Leuven, Belgium). Plasma was obtained by centrifugation for 10 min at 3000 g at 4°C and conserved at -20°C until analysis for glucose (glucose oxidase method, Thermo Fischer Scientific, Finland) and non-esterified FA (NEFA, Acyl-CoA synthase method, Wako, Sodioda, France) concentrations using an automatic analyser (ARENA 20XT, Thermo Fisher Scientific, Cergy Pontoise, France). Intra-and inter-assay coefficients of variation were 1.4 and 3.1 % for glucose and 2.1 and 3.0% for NEFA respectively.

At d10 after blood sampling, 20 F5 mothers *Azgp1*$^{+/+}$ and 20 F5 mothers *Azgp1*$^{-/-}$ were sacrificed. Individual liver, left and right abdominal mammary tissues and perigonadal adipose tissue were flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction and analyses.

2.5. RNA extraction and RT-PCR analyses

Total RNA was prepared from adipose tissue, liver and MG (about 20 mg) using PureLink RNA Mini Kit with TRIzol Reagent (Invitrogen Life Technologies, Cergy Pontoise, France) and further purified with DNase I treatment (Invitrogen Life Technologies) to eliminate contaminating genomic DNA. RNA concentrations were determined using a Nanodrop ND-1000

spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity (adipose tissue = 8.0 ± 0.8; MG = 8.7 ± 0.3; liver = 8.1 ± 0.5) was verified using a 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA).

The expression of 40 genes (Table 2) was analyzed in adipose tissue, MG and liver (n = 10 randomly chosen from each group) using TaqMan Low Density Arrays method (Applied Biosystems, Foster City, CA, USA) following the manufacturer recommendations. RT and qPCR were performed from 1µg of RNA and using the High-capacity cDNA Reverse Transcription kit and TaqMan Fast Advanced Master Mix, respectively. RT-qPCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) following conditions: 2min at 50°C and 10min at 94.5°C, followed by 40 cycles of 30s at 97°C and 1min at 59.7°C. Raw data were analyzed using Sequence Detection System Software v2.4 and Expression Suite Software v1.0.3 (Thermo Fisher Scientific, Waltham, MA USA). Relative expression of each gene was determined using the formula $2^{-\Delta\Delta Ct}$ to calculate the expression of target genes normalized to a calibrator [21]. Among the 8 endogenous control genes on the TaqMan Low Density Arrays, 5 (*ppia*, *tbp*, *eif3k*, *uxt* and *gusb*) were selected as most stable using Expression Suite Software v1.0.3 (Applied Biosystems, Foster City, CA, USA). For a given sample, the Ct data of the target genes and the geometric mean Ct of the 5 housekeeping genes were used to calculate the ΔCt. Thereafter, ΔΔCt values were calculated by subtracting the ΔCt of the calibrator (*Azgp1*$^{+/+}$ tissue) from the ΔCt of the sample (*Azgp1*$^{-/-}$ tissue). The relative quantities (RQ) were determined using the equation $RQ = 2^{-\Delta\Delta Ct}$.

Table 2
Forty selected genes used to study the effects of *Azgp1*$^{-/-}$ on adipose tissue, mammary gland and liver gene expression using TaqMan Low Density Arrays in lactating mice.

Functional category	Gene ID	Encode protein	REfSeq
FA synthesis and desaturation	*Acly*	ATP citrate lyase	NM_001199296.1
	G6pd	Glucose-6-phosphate dehydrogenase	NM_008062.2
	Me1	Malic enzyme	NM_008615.2

	Scd1	Stearoyl-Coenzyme A desaturase 1	NM_009127.4
	Scd2	Stearoyl-Coenzyme A desaturase 2	NM_009128.2
	Acaca	Acetyl-Coenzyme A carboxylase alpha	NM_133360.2
	Fasn	Fatty acid synthase	NM_007988.3
FA activation, desaturation and elongation	*Elovl5*	Elongation of very long chain fatty acids protein 5	NM_134255.3
	Acsl1	Acyl-CoA synthetase long-chain family member 1	NM_001199296.1
	Acss2	Acyl-CoA synthetase short-chain family member 2	NM_019811.3
	Fads1	Fatty acid desaturase 1	NM_146094.2
	Fads2	Fatty acid desaturase 2	NM_019699.1
Triacylglycerol synthesis	*Gpd1*	Glycerol-3-phosphate dehydrogenase	NM_010271.2
	Gpam	Glycerol-3-phosphate acyltransferase	NM_008149.3
	Dgat1	Diacylglycerol O-acyltransferase 2	NM_010046.2
	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084.2
	Pnpla2	Patatin-like phospholipase domain containing 2	NM_025802.3
FA uptake and transport	*Fabp3*	Adipocyte fatty acid binding protein 3	NM_010174.1
	Fabp4	Adipocyte fatty acid binding protein 4	NM_024406.2
	Slc27a2	Solute carrier family 27 (fatty acid transporter), member 2	NM_011978.2
	Slc27a6	Solute carrier family 27 (fatty acid transporter), member 6	NM_001081072.1
	Lpl	Lipoprotein lipase	NM_008509.2
	Cd36	CD36 molecule	NM_001159555.1
Lipolysis	*Lipe*	Lipase, Hormone-Sensitive	NM_010719.5
	Ces1	Carboxylesterase 1	NM_021456.4
Transcription factors	*Pparg*	Peroxisome proliferator activated receptor gamma	NM_011146.3
	Srebp1	Sterol regulatory element binding transcription factor 1	NM_011480.3
	Prkag2	Protein kinase, AMP-activated, gamma 2	NM_011146.3
Immune and inflammatory response	*Il6*	Interleukin 6	NM_031168.1
	Cxcl10	Chemokine (C-X-C motif) ligand 10	NM_021274.2
	Retn	Resistin	NM_001204959.1
	Saa3	Serum amyloid A 3	NM_011315.3
	Tnf	Tumor necrosis factor	NM_013693.3
Adipokines	*Adipoq*	Adiponectin	NM_009605.4
	Lep	Leptin	NM_008493.3
Major proteins in the milk fat globule membrane	*Xdh*	Xanthine dehydrogenase	NM_011723.2
	Spp1	Secreted phosphoprotein 1	NM_001204233.1
	Csn2	Casein beta	NM_009972.2
	Plin2	Perilipin 2	NM_007408.3
Glucose transporter	*Slc2a4*	Solute carrier family 2 member 4	NM_009204.2

2.6. Statistical analysis

Data concerning body weight and composition, plasma glucose and NEFA concentrations, and fat content and FA composition were analyzed using SAS, version 9.4 (SAS

Institute, Inc., Cary, NC, USA). Genotype effects on milk FA, body weight and composition at necropsy, and plasma metabolite concentrations were analyzed using ANOVA. Litter weight gain was analyzed as longitudinal data, using a mixed model with repeated measures that included the fixed effects of nursing mother genotype (*Azgp1*$^{+/+}$ *vs.* *Azgp1*$^{-/-}$), pup genotype (*Azgp1*$^{+/+}$ *vs.* *Azgp1*$^{-/-}$), the interaction of adoptive mother by litter genotype, time (*i.e.* litter age) and 2 and 3-way interactions of fixed factors with time, the random effect of nursing mother, the Kenward-Rogers adjustment for calculation of denominator degrees of freedom and ARH(1) variance-covariance structure [22]. The effects of *Azgp1*$^{-/-}$ on liver, adipose tissue and MG gene expression were analyzed with a Student's unpaired t-test using the Relative Quantification application of the Thermo Fisher Cloud software (Thermo Fisher Scientific, Waltham, MA, USA). The significance level was predefined at $P \leq 0.05$, and trends toward significance when $0.05 < P \leq 0.10$.

3. Results & Discussion

The mice genotyping was performed using PCR on all offspring confirming the absence of *Azgp1* gene.

3.1. Effects of Azgp1$^{-/-}$ *on mice during lactation*

3.1.1. Body weight

Despite all animals being fed the same standard diet, the body weight of F4 *Azgp1*$^{-/-}$ mothers was slightly (+ 7%) but significantly higher ($P < 0.01$) than *Azgp1*$^{+/+}$ mothers (Table 3A). A similar result was observed with F5 mothers at necropsy, the average body weight of *Azgp1*$^{-/-}$ was higher (+ 5%, $P < 0.05$) than *Azgp1*$^{+/+}$ mothers (Table 3B). However, body fat and lean body mass did not differ between *Azgp1*$^{+/+}$ and *Azgp1*$^{-/-}$ F5 mothers (Table 3B). The observed body weight difference is in agreement with previous research in male mice showing higher body weight gain of *Azgp1*$^{-/-}$ from 10 to 20 weeks of age, compared to the *Azgp1*$^{+/+}$

phenotype, and was explained by heavier livers in *Azgp1* $^{-/-}$ compared to *Azgp1*$^{+/+}$ mice [2]. These results are also in line with the decrease of body weight in mice overexpressing *Azgp1* [8].

Table 3

Effects of *Azgp1*$^{-/-}$ on **(A)** body weight from F4 lactating mice [aged 12-13 weeks, *Azgp1*$^{+/+}$ (n = 20) and *Azgp1*$^{-/-}$ (n = 20)] and **(B)** on body weight, body fat content and body lean content from F5 lactating mice [aged 16-18 weeks, *Azgp1*$^{+/+}$ (n = 20) and *Azgp1*$^{-/-}$ (n = 20)] at d10 of lactation and after milk sample collection. [1] Values are means ± SE. Differences were considered significant at ($P \leq 0.05$).

		***Azgp1*$^{+/+}$**	***Azgp1*$^{-/-}$**	**P <**
(A)	Body Weight (g)[1]	21.3 ± 1.6	22.8 ± 1.3	0.01
(B)	Body weight (g)[1]	35.5 ± 2.6	37.2 ± 2.5	0.04
	Body fat content (g)[1]	5.3 ± 0.8	5.6 ± 1.4	0.30
	Body lean content (g)[1]	28.5 ± 1.9	28.7 ± 1.8	0.71

3.1.2. Plasma non esterified fatty acid and glucose concentrations

There was no difference in NEFA concentrations between *Azgp1*$^{+/+}$ and *Azgp1*$^{-/-}$ lactating mice (Table 4), which is consistent with the absence of *in vivo* effects of ZAG administration during 10 days to 6-7 weeks old *ob/ob* mice on plasma NEFA concentrations [23], but contrasts with its decrease in response to ZAG administration during 5 days (half time compared to Wargent et al. [23] study) to older (20-21 weeks) *ob/ob* mice [11]. The mice age in our study is closer to that of the Russell et al [11] study but the duration of variation of ZAG quantity is longer in the case of *Azgp1*$^{-/-}$ and therefore closer to the study of Wargent et al. [22]. These different results may suggest that the duration of the changes in ZAG concentration could be important for the modulation of NEFA concentration. Glucose concentration was higher for *Azgp1*$^{-/-}$ than *Azgp1*$^{+/+}$ mothers at 10 days of lactation ($P < 0.02$, +15%; Table 4).

Table 4

Effect of *Azgp1*$^{-/-}$ on plasma glucose and NEFA concentrations of lactating mice [aged 16-18 weeks, *Azgp1*$^{+/+}$ (n = 20) and *Azgp1*$^{-/-}$ (n = 20)] at d10 of lactation and after milk sample collection. [1]Values are means ± SE. Differences were considered significant at $P \leq 0.05$.

	Azgp1$^{+/+}$	*Azgp1*$^{-/-}$	P <
Glucose (mmol/l)[1]	6.40 ± 0.79	7.36 ± 1.50	0.02
NEFA (mmol/l)[1]	0.75 ± 0.16	0.81 ± 0.18	0.32

3.1.3. Gene expression in adipose tissue

Ten genes were or tended to be downregulated in *Azgp1*$^{-/-}$ compared to *Azgp1*$^{+/+}$ F5 nursing mothers (Fig. 1; 4 genes with P < 0.05 and 6 with P < 0.10). Four of the genes play a role in FA synthesis (*Acly*, *G6pd*, *Me1* and *Scd2*), 1 in triglyceride (TG) synthesis (*Gpam)*, 2 in transport of FA and glucose (*Slc27a6* encoding FATP-2 and *Slc2a4* encoding GLUT4), 1 in FA activation (*Acss2*), and 2 genes are transcription factors (*Prkag2, Srebpf1*). These results indicate potential modifications of adipose tissue lipid metabolism in *Azgp1*$^{-/-}$ mice. The observed lower mRNA abundance of these genes is not consistent with the small but statistically significant body weight increase detected in *Azgp1*$^{-/-}$ females during lactation compared to *Azgp1*$^{+/+}$. Nonetheless, it is in agreement with the absence of body fat mass modifications in this study. The lower expression of *Slc2a4* observed in *Azgp1*$^{-/-}$ compared to *Azgp1*$^{+/+}$ mothers could potentially decrease glucose uptake by adipose tissue, and thus partially explain the increased glycaemia observed in *Azgp1*$^{-/-}$ mothers (Fig. 2). In addition, *Azgp1*$^{-/-}$ downregulated two transcriptional factors (*Srebf*, *Prkag2*) in adipose tissue that regulate the expression of lipogenic genes. The observed downregulation of *Srebf1*, an activator of lipogenic gene expression [24], is in agreement with the decreased expression of genes involved in FA synthesis (*Acly, G6pd, Me1* and *Scd2*), but contrasts with the decreased expression of *Prkag2,* which is an encoding a subunit of AMPK, AMP-activated protein kinase, that inactivates acetyl-CoA carboxylase1 [25, 26] expression. However, the AMPK signaling pathway regulates a large number of metabolic functions and compensatory mechanisms may have taken place at post-transcriptional level. No effect was detected on the expression of *Fasn*, *Lpl* and *Lipe* in adipose tissue from *Azgp1*$^{-/-}$

compared to *Azgp1*$^{+/+}$ mice, as already reported for isolated adipocytes from ZAG deficient male mice [2]. Thus, our results pointed out a potential particularity of the role of ZAG in lipid metabolism during lactation.

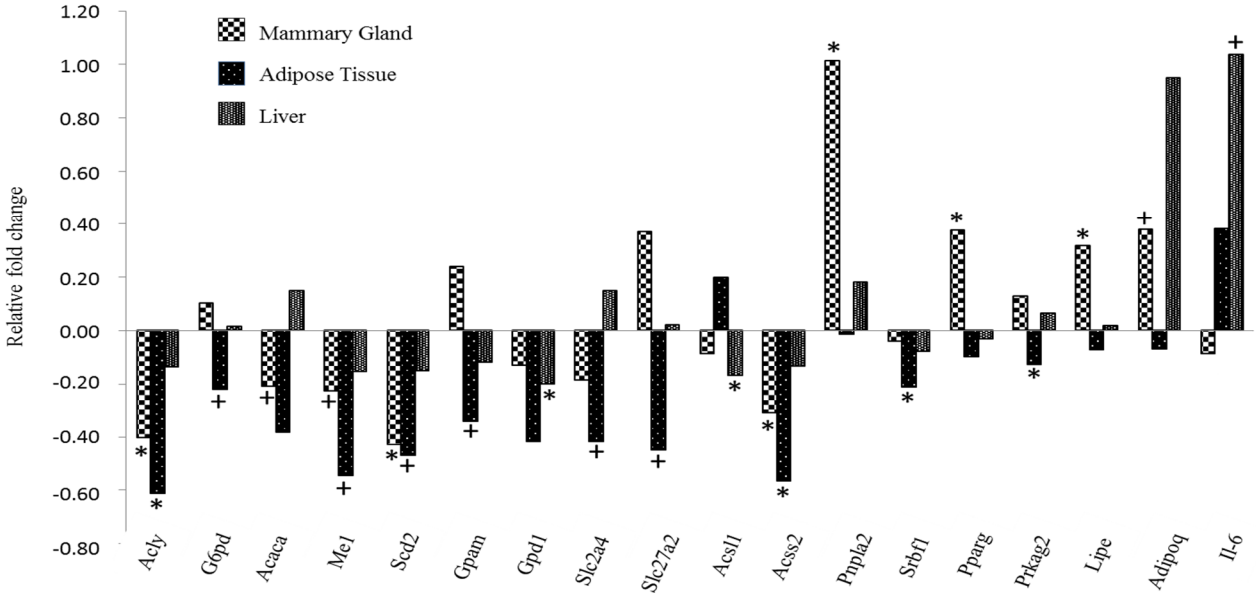


Fig. 1. Effects of *Azgp1*$^{-/-}$ on the expression of genes mostly involved in metabolism in mammary gland, adipose tissue and liver. Among the 40 studied genes, 18 genes showing a modification in their expression in at least one tissue are represented. RT-qPCR was performed on 10 *Azgp1*$^{-/-}$ and 10 *Azgp1*$^{+/+}$ lactating mice. Relative expression were calculated by $2^{-\Delta\Delta Ct}$ formula using *ppia*, *tbp*, *eif3k*, *uxt* and *gusb* as endogenous control genes. Significance: $^{+}P < 0.1$; $^{*}P \leq 0.05$.

3.1.4. Gene expression in liver

Azgp1$^{-/-}$ downregulated the expression of *Gpd1* and *Acsl1* ($P \leq 0.05$), which are genes involved in TG synthesis and FA elongation and transport, respectively (Fig. 1). The effect of *Azgp1*$^{-/-}$ in liver was limited compared to adipose tissue, because only 3 of the studied genes were differentially expressed. Previous studies showed that *AZGP1*-knockdown increased the expression of genes involved in FA synthesis (*ME1, ACACA* and *FASN*) in human hepatocytes [15, 16]. This was not observed in our study of lactating mice.

Because a link between ZAG and inflammation was established in obesity, we analyzed the expression of genes involved in immune and inflammatory response (*Il-6*, *Cxcl10*, *Retn*, *Saa3*, *Tnf* and *Lep)*. Among these genes, only *Il-6* expression was upregulated in liver of *Azgp1*$^{-/-}$ mice. Our results are consistent with the decreased *AZGP1* gene expression, and the increases protein levels of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, and MCP-1 after chronic TNF-α treatment of human adipocytes [27]. This suggests that ZAG may downregulate the expression of *IL-6* in the liver and have an anti-inflammatory effect in lactating mice. However, the effects on cytokine concentrations have to be studied in the future to confirm such hypothesis.

3.2. Effects of Azgp1$^{-/-}$ *on milk composition and litter growth*

There was no difference in milk fat content between the two genotypes (Table 5) which is in agreement with the absence of difference in plasma NEFA concentration (Table 4). The content in C16:0 (24%), C18:0 (1.7%) and C18:2n-6 (16%) in milk from *Azgp1*$^{+/+}$ and *Azgp1*$^{-/-}$ mice (Table 5) is consistent with those observed in C57BI/6J mice at d10 of lactation corresponding to 24%, 2% and 11% in the latter, respectively[28]. The sum of saturated FA tended to be lower in *Azgp1*$^{-/-}$ than *Azgp1*$^{+/+}$ (C7 to C25; - 6%; $P < 0.06$). Two minor FAs, C15:0 and C17:0, were present in higher concentrations in the milk from *Azgp1*$^{-/-}$ compared to *Azgp1*$^{+/+}$ mice (+11% and +7 %, respectively; $P < 0.03$), whereas C14:0 and C16:0 (corresponding to the major medium chain FAs) tended to be lower in *Azgp1* $^{-/-}$ milk. The decrease of C14:0 and C16:0 was also observed after a knockout of *Fasn*, a well-known lipogenic gene in mouse mammary epithelial cells [28].

Regarding the unsaturated FA, a higher concentration of the sum of polyunsaturated FA (+ 6%, $P < 0.01$) was observed in milk from *Azgp1*$^{-/-}$ mice, but no effect was observed on the sum of monounsaturated FA (C 12:1 to C24:1) concentration. The sum of n-3 polyunsaturated

312 FA (+ 5 %, P < 0.02) was increased in *Azgp1*$^{-/-}$ compared with *Azgp1*$^{+/+}$ milk, and particularly the
313 C18:3n-3 (+ 6%, P < 0.01). The sum of n-6 polyunsaturated FA (+ 6%, P < 0.01) also increased,
314 which was mainly due to C18:2n-6 (+ 6%, P < 0.01), 20:2n-6 (+ 12%, P < 0.04) and 20:3n-6 (+
315 9%, P < 0.02). The sum of conjugated linoleic acid (CLA) concentration tended to be higher in
316 *Azgp1*$^{-/-}$ than *Azgp1*$^{+/+}$ reflecting the higher content of *cis*-9, *cis*-11 CLA isomer (+ 26%, P < 0.02)
317 in milk from *Azgp1*$^{-/-}$ mice. Consequently, our study suggested an effect of *Azgp1*$^{-/-}$ on milk FA
318 composition.

319 **Table 5**
320 Effect of *Azgp1*$^{-/-}$ on fat content and fatty acid composition of milk from lactating mice (aged
321 16-18 weeks, *Azgp1*$^{+/+}$ (n = 19) and *Azgp1*$^{-/-}$, (n = 20)) at d9 of lactation. Values are means ± SE.
322 In bold fatty acid (FA) with P ≤ 0.05 and in italic bold FA with 0.05 < P ≤ 0.1.

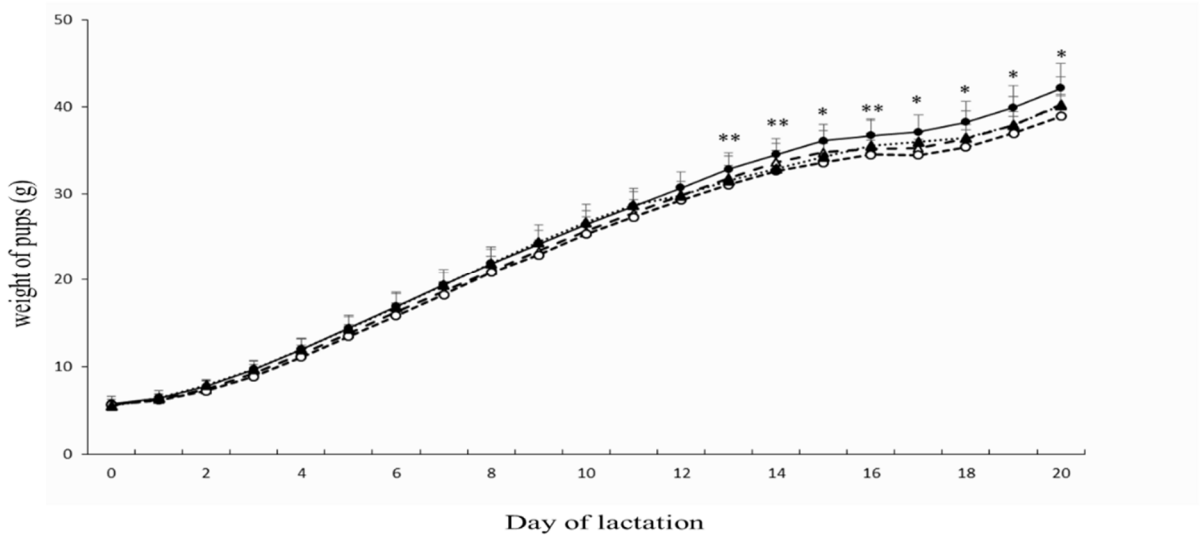
	***Azgp1*$^{+/+}$**	***Azgp1*$^{-/-}$**	**P <**
Fat (g/100 g milk)	30.80 ± 2.14	29.79 ± 3.53	0.28
FA composition (g/100g FA)			
C15:0	**0.114 ± 0.014**	**0.127 ± 0.020**	0.03
C17:0	**0.095 ± 0.009**	**0.102 ± 0.011**	0.03
C14:0	*12.87 ± 1.95*	*11.67 ± 2.17*	*0.08*
C16:0	*24.41 ± 1.62*	*23.60 ± 1.25*	*0.09*
C18:0	1.705 ± 0.141	1.759 ± 0.262	0.42
C20:0	0.056 ± 0.006	0.057 ± 0.007	0.54
C22:0	0.949 ± 0.149	1.024 ± 0.169	0.15
C24:0	0.045 ± 0.012	0.047 ± 0.018	0.63
C18:2n-6	**15.59 ± 1.10**	**16.54 ± 0.94**	0.01
C18:3n-3	**1.000 ± 0.079**	**1.056 ± 0.107**	0.01
C18:3n-6	0.209 ± 0.042	0.209 ± 0.037	0.94
C20:2n-6	**1.125 ± 0.187**	**1.262 ± 0.212**	0.04
C20:3n-6	**0.142 ± 0.014**	**0.155 ± 0.018**	0.02
C20:5n-3	0.240 ± 0.028	0.245 ± 0.021	0.47
C22:3n-3	0.014 ± 0.006	0.013 ± 0.004	0.61
C22:6n-3	*0.397 ± 0.047*	*0.423 ± 0.040*	*0.06*
C22:2n-6	0.104 ± 0.038	0.108 ± 0.019	0.61
C22:4n-6	0.327 ± 0.070	0.337 ± 0.074	0.64
cis-9 C18:1	18.00 ± 2.53	19.52 ± 3.33	0.12
trans-10 C18:1	0.005 ± 0.004	0.006 ± 0.005	0.59
trans-11 C18:1	0.010 ± 0.005	0.010 ± 0.006	1.00
cis-9, cis-11 CLA	**0.035 ± 0.014**	**0.044 ± 0.013**	0.02

cis-9, trans-11 CLA	0.023 ± 0.008	0.027 ± 0.012	0.25
trans-11, trans-13 CLA	0.035 ± 0.006	0.036 ± 0.007	0.71
trans-9, trans-11 CLA	0.049 ± 0.009	0.054 ± 0.014	0.24
∑ Saturated	*54.15 ± 4.45*	*50.96 ± 5.59*	0.06
∑ Monounsaturated	23.99 ± 3.24	25.85 ± 4.51	0.15
∑ Polyunsaturated	**20.40 ± 1.42**	**21.68 ± 1.30**	0.01
∑ Polyunsaturated n-3	**2.85 ± 0.20**	**3.00 ± 0.18**	0.02
∑ Polyunsaturated n-6	**17.49 ± 1.29**	**18.62 ± 1.15**	0.01
∑ CLA	*0.14 ± 0.22*	*0.16 ± 0.03*	0.08

Weight of each litter composed of 4 pups was recorded from 0 to 20 days of age (Fig. 2, supplementary Table 1). *Azgp1*$^{+/+}$ litters nursed by mothers carrying the same genotype (n = 9) weighted 5.7 ± 0.3 g, 25.4 ± 1.9 g and 38.9 ± 2.5 g at d0, d10 and d20 postpartum, respectively. The weight of *Azgp1* $^{-/-}$ litters with mothers carrying the same genotype (n = 8) was 5.5 ± 0.2 g, 26.7 ± 2.1 g and 40.2 ± 3.3 g at d0, d10 and d20, respectively. The weight of *Azgp1*$^{-/-}$ litters nursed by *Azgp1*$^{+/+}$ mothers (n =13) was 5.1 ± 0.2 g, 25.7 ± 2.4 g and 40.3 ± 3.1 g at d0, d10 and d20, respectively. The litter weight *Azgp1*$^{+/+}$ litters nursed by *Azgp1*$^{-/-}$ mothers (n =13) was 5.4 ± 1.5 g, 24.7 ± 3.7 g and 42.2 ± 2.7 g at d0, d10 and d20 postpartum, respectively. Significant interactions were observed for litter growth between adoptive mother genotype and day ($P < 0.006$), and between adoptive mother genotype, pup genotype and day ($P < 0.001$). All *Azgp1*$^{-/-}$ pups showed similar growth rates regardless of the nursing mother's genotype. In contrast and interestingly, the growth of *Azgp1*$^{+/+}$ pups was significantly higher when nursed by *Azgp1*$^{-/-}$ than *Azgp1*$^{+/+}$ mothers, but only after d13 (Fig. 2). Although the weight differences are very small, the higher growth of *Azgp1*$^{+/+}$ pups may have been mediated by milk fatty acid composition of *Azgp1*$^{-/-}$ mice. As we showed, changes in milk FA composition (in particular an increase in polyunsaturated FA) from *Azgp1*$^{-/-}$, we could suggest that these modifications led to a faster growth of *Azgp1*$^{+/+}$ pup. The effect of milk FA composition modifications on pup growth rate has been previously reported in *Fasn* knockout mice showing a decrease of C18:0 and C18:2 content, and total mice milk FAs, leading to a slower growth of pups from *Fasn*$^{-/-}$ compared to

wild-type mothers [29]. However, it cannot be excluded that *Azgp1*$^{-/-}$ mothers produced more milk than *Azgp1*$^{+/+}$ mothers influencing only *Azgp1*$^{+/+}$ pups or that the knockout could affect other milk components such as peptides or miRNA affecting the growth of pups.

Fig. 2. Effect of cross-feeding on the growth pups nursed by adoptive mothers from d0 until d20 postpartum. Pups were separated from their respective mothers and swapped within 24 h after birth. This litter swap allowed to have 9 mothers $^{+/+}$ with pups $^{+/+}$ (white circle), 13 mothers $^{+/+}$ with pups $^{-/-}$ (white triangle), 13 mothers $^{-/-}$ with pups $^{+/+}$ (black circle), 8 mothers $^{-/-}$ with pups $^{-/-}$ (black triangle), with an adjustment of 4 pups per mother. The litter was weighed daily from d0 until d20 postpartum. Mother genotype × Day ($P < 0.006$); Mother × Pup × Day ($P < 0.001$) and comparison Mother $^{+/+}$ × pups $^{+/+}$ *vs.* Mother $^{-/-}$ × pups $^{+/+}$: $^{*}P < 0.05$; $^{**}P < 0.01$.



3.3. *Effects of Azgp1*$^{-/-}$ on mammary gland gene expression

To better understand molecular mechanisms underlying the effects of *Azgp1*$^{-/-}$ on milk FA composition, we analyzed the expression of 40 genes in MG in F5 nursing mothers (Table 2). *Azgp1*$^{-/-}$ altered the expression of 9 genes in the MG, including four common genes between MG and adipose tissue (Fig. 1; Supplementary Fig. 3). Azgp1$^{-/-}$ increased the expression of *Pnpla2* and *Lipe* ($P < 0.05$), encoding respectively for adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (Fig. 1). These genes encode two major lipases [30], and their

upregulation suggests compensatory mechanisms to ensure lipolysis in the absence of ZAG. Such effect would explain in part the observed increase of some long chain FA in milk, such as C18:2n-6, C18:3n-3, and C20:2n-6.

We observed a downregulation of 5 genes involved in *de novo* lipid synthesis (*Acly*, *Scd2*, *and Acss2* with $P < 0.05$ and *Me1*, *Acaca* with $P < 0.10$), which is consistent with the decreased C14:0 and C16:0 content in *Azgp1*$^{-/-}$ mice. However, the downregulation of *scd2* is not consistent with the increase of the sum of unsaturated FA in milk. *Scd2* gene is a member of a desaturase family, and other members could be responsible for this increase. In addition, the increased of *Pparg* expression in *Azgp1*$^{-/-}$ mice, a gene known to regulate the lipogenesis, does not agree with the decrease of the expression of other lipogenic genes. Such discrepancy could be due to the action of other regulatory mechanisms, for instance via miRNA. For example, *PPARG* mRNA is a direct target of miR-130a which modifies its expression in bovine mammary epithelial cells [31]. Moreover, we studied the expression of only 40 mRNA and cannot exclude that the expression of other genes could have been influenced by *Azgp1*$^{-/-}$.

4. Conclusion

We found that *Azgp1*$^{-/-}$ downregulated expression of genes involved in lipid metabolism, particularly in FA synthesis and transport, and TG synthesis in adipose tissue and MG, and to a lesser extent, in liver of lactating mice. The downregulation of lipid synthesis pathways in adipose tissue suggests a particular role of *Azgp1* in lipid metabolism during lactation. Altered MG lipid metabolism could explain in part the modifications of milk FA composition observed in this study, and therefore the higher growth of *Azgp1*$^{+/+}$ pups nursed by *Azgp1*$^{-/-}$ mothers.

Supplementary Figure and Table Legends

The following section are available online. **Supplementary Fig. 1:** Design of the transgene to create *Azgp1*$^{-/-}$ mice. The targeting vector was constructed by replacing the 8 exons of the *Azgp1* gene, with a neomycin resistance (Neo) gene and LacZ reporter gene. The sequences of the primers used are presented in Table 1. **Supplementary Fig. 2:** Representative scheme explaining the establishment of the *Azgp1*$^{-/-}$ mouse line. **Supplementary Fig. 3**: Venn diagram of genes differentially expressed in mammary, hepatic and adipose tissues in 10 *Azgp1*$^{-/-}$ versus 10 *Azgp1*$^{+/+}$ lactating mice. Expression analyses were performed by quantitative RT-qPCR using a TaqMan Low Density Arrays. **Supplementary Table 1**: Effects of cross-feeding on pup weight. Mean of 4 pups per litter and SE are indicated from day 0 to day 20 after birth.

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Author Contributions

Y.F and C.Le contributed to the design, performing of the study and data interpretation. C.La was in charge of monitoring of the animals. C.B performed TLDA analyses. J.P performed

statistical analyses of phenotypic data. Y.F and C.Le wrote the main manuscript and J.P and C.B improved it. All authors reviewed the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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