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1 **Effects of *Azgp1*^{-/-} on mammary gland, adipose tissue and**
2 **liver gene expression and milk lipid composition in lactating**
3 **mice**

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18

19 **Abstract**

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

35

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

38

39

40 **1. Introduction**

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

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

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

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

77

78 **2. Materials and methods**

79 *2.1. Generation of the *Azgp1*^{-/-} mice.*

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

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

107 **Table 1**

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

109

Target	Forward sequence	Reverse sequence
Azgp1	TGGCTCGGTTGAGAGGATG	ACTTGGTTTATGTGGTGCTTAGG
LacZ	GGTAAACTGGCTCGGATTAGGG	TTGACTGTAGCGGCTGATGTTG

110 **2.2. Litter growth, mother body weight and body composition**

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

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

122

123 *2.3. Milk sampling and analyses*

124 At mid-lactation (d9), 20 *Azgp1*^{+/+} and 20 *Azgp1*^{-/-} mothers of F5 generation were
125 separated from the litter for 2 hours before collection of milk samples, injected with 0.3 U
126 synthetic oxytocin (CEVA Sante Animale, Libourne, France) and then anesthetized by
127 intraperitoneal injection (0.01 mL/g of body weight) of a solution containing 1 mL Imalgène
128 1000 (Merial, Lyon, France), 0.6 mL Rompun (Bayer Pharma, Puteaux, France) in a final
129 volume of 10 mL water. The MG was then massaged slightly to induce milk secretion into a
130 hematocrit tube. Fat content in fresh milk was determined by the creamatocrit method [19].
131 Approximately 75 µl of whole milk was drawn into a standard glass capillary tube (75 mm in
132 length, 1.5 mm and 1.2 mm in outside and inside diameters, respectively), centrifuged at 12,000
133 rpm for 12 min, and fat content was calculated by the following formula: $Y = [(X - 0.59)/0.146]$
134 $\times 0.1$, where Y = fat content (g/100 ml) and X = percentage of cream length total sample in the
135 tube (creamatocrit percentage). Fat samples obtained by capillary tube centrifugation were
136 analyzed for FA composition after a one-step extraction and methylation. The FA Methyl Ester
137 (FAME) profile was determined using a Trace-GC 2000 Series gas chromatograph and 0.6 µL of
138 sample and a split ratio of 1:30, as previously described for FAME analysis of milk [20].

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

141

142 *2.4. Blood and tissue sampling*

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

146 After anesthesia, blood samples were immediately drawn from the periorbital sinus of
147 eyes at d10 into evacuated tubes containing EDTA (1.95 mg/mL; Terumo Europe NV, Leuven,
148 Belgium). Plasma was obtained by centrifugation for 10 min at 3000 g at 4°C and conserved at -
149 20°C until analysis for glucose (glucose oxidase method, Thermo Fischer Scientific, Finland)
150 and non-esterified FA (NEFA, Acyl-CoA synthase method, Wako, Sodioda, France)
151 concentrations using an automatic analyser (ARENA 20XT, Thermo Fisher Scientific, Cergy
152 Pontoise, France). Intra-and inter-assay coefficients of variation were 1.4 and 3.1 % for glucose
153 and 2.1 and 3.0% for NEFA respectively.

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

158

159 *2.5. RNA extraction and RT-PCR analyses*

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

164 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity (adipose tissue
 165 = 8.0 ± 0.8 ; MG = 8.7 ± 0.3 ; liver = 8.1 ± 0.5) was verified using a 2100 Bioanalyzer (Agilent
 166 Technologies, Foster City, CA, USA).

167 The expression of 40 genes (Table 2) was analyzed in adipose tissue, MG and liver (n =
 168 10 randomly chosen from each group) using TaqMan Low Density Arrays method (Applied
 169 Biosystems, Foster City, CA, USA) following the manufacturer recommendations. RT and qPCR
 170 were performed from 1µg of RNA and using the High-capacity cDNA Reverse Transcription kit
 171 and TaqMan Fast Advanced Master Mix, respectively. RT-qPCR amplification was performed
 172 using an Applied Biosystems Prism 7900HT sequence detection system (Applied Biosystems,
 173 Foster City, CA, USA) following conditions: 2min at 50°C and 10min at 94.5°C, followed by 40
 174 cycles of 30s at 97°C and 1min at 59.7°C. Raw data were analyzed using Sequence Detection
 175 System Software v2.4 and Expression Suite Software v1.0.3 (Thermo Fisher Scientific,
 176 Waltham, MA USA). Relative expression of each gene was determined using the formula $2^{-\Delta\Delta Ct}$
 177 to calculate the expression of target genes normalized to a calibrator [21]. Among the 8
 178 endogenous control genes on the TaqMan Low Density Arrays, 5 (*ppia*, *tbp*, *eif3k*, *uxt* and *gusb*)
 179 were selected as most stable using Expression Suite Software v1.0.3 (Applied Biosystems, Foster
 180 City, CA, USA). For a given sample, the Ct data of the target genes and the geometric mean Ct
 181 of the 5 housekeeping genes were used to calculate the ΔCt . Thereafter, $\Delta\Delta Ct$ values were
 182 calculated by subtracting the ΔCt of the calibrator (*Azgp1*^{+/+} tissue) from the ΔCt of the sample
 183 (*Azgp1*^{-/-} tissue). The relative quantities (RQ) were determined using the equation $RQ = 2^{-\Delta\Delta Ct}$.

184 **Table 2**

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

187

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

	<i>Scd1</i>	Stearoyl-Coenzyme A desaturase 1	NM_009127.4
	<i>Scd2</i>	Stearoyl-Coenzyme A desaturase 2	NM_009128.2
	<i>Acaca</i>	Acetyl-Coenzyme A carboxylase alpha	NM_133360.2
	<i>Fasn</i>	Fatty acid synthase	NM_007988.3
FA activation, desaturation and elongation	<i>Elovl5</i>	Elongation of very long chain fatty acids protein 5	NM_134255.3
	<i>Acs11</i>	Acyl-CoA synthetase long-chain family member 1	NM_001199296.1
	<i>Acss2</i>	Acyl-CoA synthetase short-chain family member 2	NM_019811.3
	<i>Fads1</i>	Fatty acid desaturase 1	NM_146094.2
	<i>Fads2</i>	Fatty acid desaturase 2	NM_019699.1
Triacylglycerol synthesis	<i>Gpd1</i>	Glycerol-3-phosphate dehydrogenase	NM_010271.2
	<i>Gpam</i>	Glycerol-3-phosphate acyltransferase	NM_008149.3
	<i>Dgat1</i>	Diacylglycerol O-acyltransferase 2	NM_010046.2
	<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084.2
	<i>Pnpla2</i>	Patatin-like phospholipase domain containing 2	NM_025802.3
FA uptake and transport	<i>Fabp3</i>	Adipocyte fatty acid binding protein 3	NM_010174.1
	<i>Fabp4</i>	Adipocyte fatty acid binding protein 4	NM_024406.2
	<i>Slc27a2</i>	Solute carrier family 27 (fatty acid transporter), member 2	NM_011978.2
	<i>Slc27a6</i>	Solute carrier family 27 (fatty acid transporter), member 6	NM_001081072.1
	<i>Lpl</i>	Lipoprotein lipase	NM_008509.2
	<i>Cd36</i>	CD36 molecule	NM_001159555.1
Lipolysis	<i>Lipe</i>	Lipase, Hormone-Sensitive	NM_010719.5
	<i>Ces1</i>	Carboxylesterase 1	NM_021456.4
Transcription factors	<i>Pparg</i>	Peroxisome proliferator activated receptor gamma	NM_011146.3
	<i>Srebp1</i>	Sterol regulatory element binding transcription factor 1	NM_011480.3
	<i>Prkag2</i>	Protein kinase, AMP-activated, gamma 2	NM_011146.3
Immune and inflammatory response	<i>Il6</i>	Interleukin 6	NM_031168.1
	<i>Cxcl10</i>	Chemokine (C-X-C motif) ligand 10	NM_021274.2
	<i>Retn</i>	Resistin	NM_001204959.1
	<i>Saa3</i>	Serum amyloid A 3	NM_011315.3
	<i>Tnf</i>	Tumor necrosis factor	NM_013693.3
Adipokines	<i>Adipoq</i>	Adiponectin	NM_009605.4
	<i>Lep</i>	Leptin	NM_008493.3
Major proteins in the milk fat globule membrane	<i>Xdh</i>	Xanthine dehydrogenase	NM_011723.2
	<i>Spp1</i>	Secreted phosphoprotein 1	NM_001204233.1
	<i>Csn2</i>	Casein beta	NM_009972.2
	<i>Plin2</i>	Perilipin 2	NM_007408.3
Glucose transporter	<i>Slc2a4</i>	Solute carrier family 2 member 4	NM_009204.2

188

189 2.6. Statistical analysis

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

192 Institute, Inc., Cary, NC, USA). Genotype effects on milk FA, body weight and composition at
193 necropsy, and plasma metabolite concentrations were analyzed using ANOVA. Litter weight
194 gain was analyzed as longitudinal data, using a mixed model with repeated measures that
195 included the fixed effects of nursing mother genotype (*Azgp1^{+/+}* vs. *Azgp1^{-/-}*), pup genotype
196 (*Azgp1^{+/+}* vs. *Azgp1^{-/-}*), the interaction of adoptive mother by litter genotype, time (*i.e.* litter age)
197 and 2 and 3-way interactions of fixed factors with time, the random effect of nursing mother, the
198 Kenward-Rogers adjustment for calculation of denominator degrees of freedom and ARH(1)
199 variance-covariance structure [22]. The effects of *Azgp1^{-/-}* on liver, adipose tissue and MG gene
200 expression were analyzed with a Student's unpaired t-test using the Relative Quantification
201 application of the Thermo Fisher Cloud software (Thermo Fisher Scientific, Waltham, MA,
202 USA). The significance level was predefined at $P \leq 0.05$, and trends toward significance when
203 $0.05 < P \leq 0.10$.

204

205 **3. Results & Discussion**

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

208 ***3.1. Effects of *Azgp1^{-/-}* on mice during lactation***

209 **3.1.1. Body weight**

210 Despite all animals being fed the same standard diet, the body weight of F4 *Azgp1^{-/-}*
211 mothers was slightly (+ 7%) but significantly higher ($P < 0.01$) than *Azgp1^{+/+}* mothers (Table
212 3A). A similar result was observed with F5 mothers at necropsy, the average body weight of
213 *Azgp1^{-/-}* was higher (+ 5%, $P < 0.05$) than *Azgp1^{+/+}* mothers (Table 3B). However, body fat and
214 lean body mass did not differ between *Azgp1^{+/+}* and *Azgp1^{-/-}* F5 mothers (Table 3B). The
215 observed body weight difference is in agreement with previous research in male mice showing
216 higher body weight gain of *Azgp1^{-/-}* from 10 to 20 weeks of age, compared to the *Azgp1^{+/+}*

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

219

220 **Table 3**

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

226

		<i>Azgp1</i> ^{+/+}	<i>Azgp1</i> ^{-/-}	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

227

228 **3.1.2. Plasma non esterified fatty acid and glucose concentrations**

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

239

240 **Table 4**

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

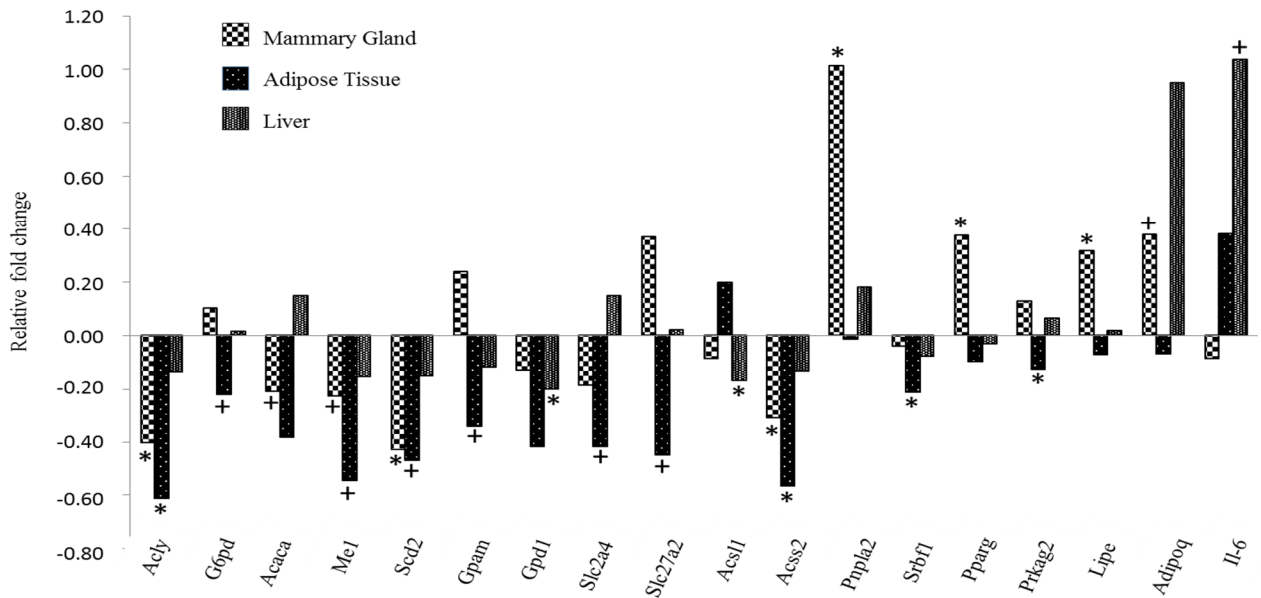
	<i>Azgp1</i> ^{+/+}	<i>Azgp1</i> ^{-/-}	P <	244 245
Glucose (mmol/l) ¹	6.40 ± 0.79	7.36 ± 1.50	0.02	
NEFA (mmol/l) ¹	0.75 ± 0.16	0.81 ± 0.18	0.32	246

247

248 3.1.3. Gene expression in adipose tissue

249 Ten genes were or tended to be downregulated in *Azgp1*^{-/-} compared to *Azgp1*^{+/+} F5
250 nursing mothers (Fig. 1; 4 genes with P < 0.05 and 6 with P < 0.10). Four of the genes play a role
251 in FA synthesis (*Acly*, *G6pd*, *Me1* and *Scd2*), 1 in triglyceride (TG) synthesis (*Gpam*), 2 in
252 transport of FA and glucose (*Slc27a6* encoding FATP-2 and *Slc2a4* encoding GLUT4), 1 in FA
253 activation (*Acss2*), and 2 genes are transcription factors (*Prkag2*, *Srebp1*). These results indicate
254 potential modifications of adipose tissue lipid metabolism in *Azgp1*^{-/-} mice. The observed lower
255 mRNA abundance of these genes is not consistent with the small but statistically significant
256 body weight increase detected in *Azgp1*^{-/-} females during lactation compared to *Azgp1*^{+/+}.
257 Nonetheless, it is in agreement with the absence of body fat mass modifications in this study.
258 The lower expression of *Slc2a4* observed in *Azgp1*^{-/-} compared to *Azgp1*^{+/+} mothers could
259 potentially decrease glucose uptake by adipose tissue, and thus partially explain the increased
260 glycaemia observed in *Azgp1*^{-/-} mothers (Fig. 2). In addition, *Azgp1*^{-/-} downregulated two
261 transcriptional factors (*Srebf*, *Prkag2*) in adipose tissue that regulate the expression of lipogenic
262 genes. The observed downregulation of *Srebf1*, an activator of lipogenic gene expression [24], is
263 in agreement with the decreased expression of genes involved in FA synthesis (*Acly*, *G6pd*, *Me1*
264 and *Scd2*), but contrasts with the decreased expression of *Prkag2*, which is an encoding a subunit
265 of AMPK, AMP-activated protein kinase, that inactivates acetyl-CoA carboxylase1 [25, 26]
266 expression. However, the AMPK signaling pathway regulates a large number of metabolic
267 functions and compensatory mechanisms may have taken place at post-transcriptional level. No
268 effect was detected on the expression of *Fasn*, *Lpl* and *Lipe* in adipose tissue from *Azgp1*^{-/-}

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



272
 273 **Fig. 1.** Effects of *Azgp1*^{-/-} on the expression of genes mostly involved in metabolism in
 274 mammary gland, adipose tissue and liver. Among the 40 studied genes, 18 genes showing a
 275 modification in their expression in at least one tissue are represented. RT-qPCR was performed
 276 on 10 *Azgp1*^{-/-} and 10 *Azgp1*^{+/+} lactating mice. Relative expression were calculated by 2^{-ΔΔCt}
 277 formula using *ppia*, *tbp*, *eif3k*, *uxt* and *gusb* as endogenous control genes. Significance: *P < 0.1;
 278 * P ≤ 0.05.

279

280 3.1.4. Gene expression in liver

281 *Azgp1*^{-/-} downregulated the expression of *Gpd1* and *Acsl1* (P ≤ 0.05), which are genes
 282 involved in TG synthesis and FA elongation and transport, respectively (Fig. 1). The effect of
 283 *Azgp1*^{-/-} in liver was limited compared to adipose tissue, because only 3 of the studied genes
 284 were differentially expressed. Previous studies showed that *AZGP1*-knockdown increased the
 285 expression of genes involved in FA synthesis (*ME1*, *ACACA* and *FASN*) in human hepatocytes
 286 [15, 16]. This was not observed in our study of lactating mice.

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

296

297 **3.2. Effects of *Azgp1^{-/-}* on milk composition and litter growth**

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

309 Regarding the unsaturated FA, a higher concentration of the sum of polyunsaturated FA
310 (+ 6%, P < 0.01) was observed in milk from *Azgp1^{-/-}* mice, but no effect was observed on the
311 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.

	<i>Azgp1^{+/+}</i>	<i>Azgp1^{-/-}</i>	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
<i>C14:0</i>	<i>12.87 ± 1.95</i>	<i>11.67 ± 2.17</i>	<i>0.08</i>
<i>C16:0</i>	<i>24.41 ± 1.62</i>	<i>23.60 ± 1.25</i>	<i>0.09</i>
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
<i>C22:6n-3</i>	<i>0.397 ± 0.047</i>	<i>0.423 ± 0.040</i>	<i>0.06</i>
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

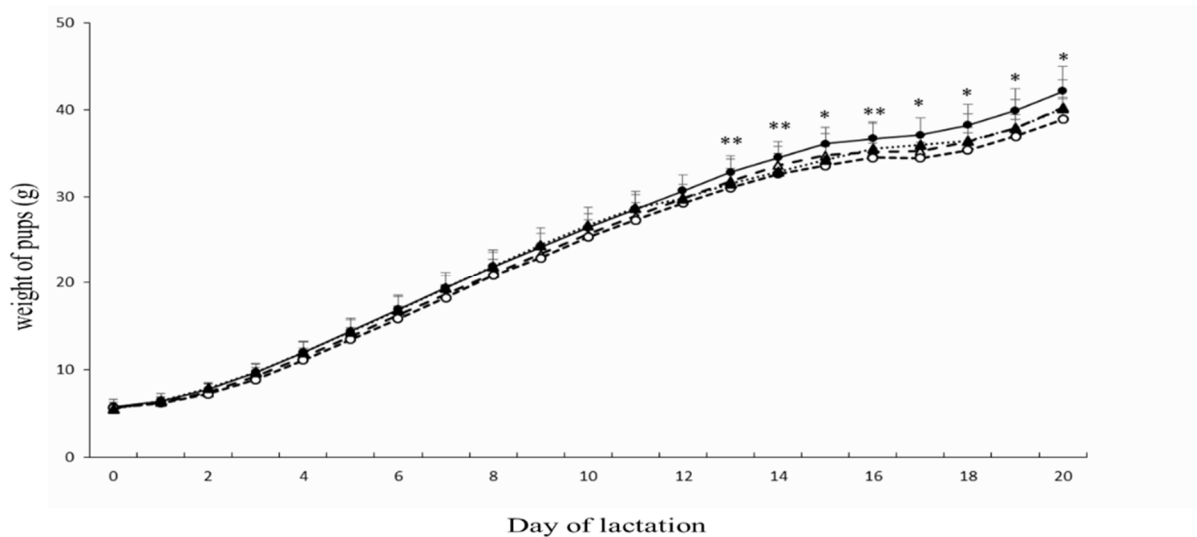
cis-9, trans-11 CLA	0.023 ± 0.008	0.027 ± 0.012	0.25
trans-11, trans-13 CLA	0.035 ± 0.006	0.036 ± 0.007	0.71
trans-9, trans-11 CLA	0.049 ± 0.009	0.054 ± 0.014	0.24
∑ Saturated	54.15 ± 4.45	50.96 ± 5.59	0.06
∑ Monounsaturated	23.99 ± 3.24	25.85 ± 4.51	0.15
∑ Polyunsaturated	20.40 ± 1.42	21.68 ± 1.30	0.01
∑ 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

323
324 Weight of each litter composed of 4 pups was recorded from 0 to 20 days of age (Fig. 2,
325 supplementary Table 1). *Azgp1^{+/+}* litters nursed by mothers carrying the same genotype (n = 9)
326 weighted 5.7 ± 0.3 g, 25.4 ± 1.9 g and 38.9 ± 2.5 g at d0, d10 and d20 postpartum, respectively.
327 The weight of *Azgp1^{-/-}* litters with mothers carrying the same genotype (n = 8) was 5.5 ± 0.2 g,
328 26.7 ± 2.1 g and 40.2 ± 3.3 g at d0, d10 and d20, respectively. The weight of *Azgp1^{-/-}* litters
329 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
330 d20, respectively. The litter weight *Azgp1^{+/+}* litters nursed by *Azgp1^{-/-}* mothers (n = 13) was 5.4 ±
331 1.5 g, 24.7 ± 3.7 g and 42.2 ± 2.7 g at d0, d10 and d20 postpartum, respectively. Significant
332 interactions were observed for litter growth between adoptive mother genotype and day (P <
333 0.006), and between adoptive mother genotype, pup genotype and day (P < 0.001). All *Azgp1^{-/-}*
334 pups showed similar growth rates regardless of the nursing mother's genotype. In contrast and
335 interestingly, the growth of *Azgp1^{+/+}* pups was significantly higher when nursed by *Azgp1^{-/-}* than
336 *Azgp1^{+/+}* mothers, but only after d13 (Fig. 2). Although the weight differences are very small, the
337 higher growth of *Azgp1^{+/+}* pups may have been mediated by milk fatty acid composition of
338 *Azgp1^{-/-}* mice. As we showed, changes in milk FA composition (in particular an increase in
339 polyunsaturated FA) from *Azgp1^{-/-}*, we could suggest that these modifications led to a faster
340 growth of *Azgp1^{+/+}* pup. The effect of milk FA composition modifications on pup growth rate
341 has been previously reported in *Fasn* knockout mice showing a decrease of C18:0 and C18:2
342 content, and total mice milk FAs, leading to a slower growth of pups from *Fasn^{-/-}* compared to

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

346
347 **Fig. 2.** Effect of cross-feeding on the growth pups nursed by adoptive mothers from d0 until d20
348 postpartum. Pups were separated from their respective mothers and swapped within 24 h after
349 birth. This litter swap allowed to have 9 mothers ^{+/+} with pups ^{+/+} (white circle), 13 mothers ^{+/+}
350 with pups ^{-/-} (white triangle), 13 mothers ^{-/-} with pups ^{+/+} (black circle), 8 mothers ^{-/-} with pups ^{-/-}
351 (black triangle), with an adjustment of 4 pups per mother. The litter was weighed daily from d0
352 until d20 postpartum. Mother genotype × Day (P < 0.006); Mother × Pup × Day (P < 0.001) and
353 comparison Mother ^{+/+} × pups ^{+/+} vs. Mother ^{-/-} × pups ^{+/+}: *P < 0.05; **P < 0.01.

354



355

356

357 3.3. Effects of *Azgp1*^{-/-} on mammary gland gene expression

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

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

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

378

379 **4. Conclusion**

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

386

387 **Supplementary Figure and Table Legends**

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

397

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408

409 **Author Contributions**

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

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

414

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419 **Conflict of interest**

420 The authors declare no conflict of interest.

421

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