A gene and protein expression study on four porcine genes related to intramuscular fat deposition

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Abstract

Intramuscular fat (IMF) content has a prominent role in meat quality, affecting sensory attributes such as flavour and texture. In the present research, we studied in samples of porcine *Semimembranosus* muscle four genes related to lipid metabolism and whose gene expressions have been associated to IMF deposition: *FASN*, *SCD*, *LIPE* and *LPL*. We analysed both mRNA and protein expressions in two groups of Italian Large White pigs divergent for *Semimembranosus* IMF deposition, with the aim of comparing the levels of four genes and enzymes between the two groups and identifying possible coexpression links. The obtained results suggest a prominent role of LIPE enzyme in IMF hydrolysis, as the samples with low IMF deposition show a significantly higher amount of this lipase. Finally, a poorly known correlation was found between LIPE and FASN enzymes only in female individuals. These results provide new information for the understanding of IMF deposition.

1 Introduction

During the last decades, pig selection has aimed at satisfying the request of the pork industry mainly focused on the increase of muscle deposition and carcass lean cut amount, resulted in a reduction in fat storage and adipogenesis (Wood, 1990). This selective pressure has also led to a progressive lowering in the total lipid content of muscle, in particular in some breeds selected for their predisposition to lean mass deposition (Wood and Warriss, 1992). In Large White breed the selection lowered noticeably the marbling percentage, passing from an average of 2-4% of intramuscular fat (IMF) in Large White pigs bred in 1960's (Wood, 1990) to less than 1% in *Longissimus* muscle of the modern Large White pigs (Wood *et al.*, 2008). IMF is composed of lipid droplets stored within myofibers cytoplasm and adipocytes located between the fiber *fasciculi*, consequently IMF amount is strongly related to the number of intramuscular adipocytes (Zheng and Mei, 2009). It is known that sensory attributes such as flavour and juiciness are influenced by IMF content (Wood, 1990; Fernandez et al., 1999).

The number of Genome Wide Association studies aimed at identifying SNPs and genes affecting IMF deposition and composition in different pig breeds (Ma et al., 2013; Muñoz et al., 2013; Nonneman et al., 2013; Kim et al., 2015; Davoli et al., 2016) is increasing, however little is known about the relative enzyme quantity of the putative genes involved in IMF deposition in pig muscle tissue. Therefore, in the present study we considered four genes involved in lipid metabolism and whose mRNA levels in literature are reported to be linked to IMF deposition in different pig breeds (Zhao et al., 2009; Wang et al., 2012). Two of the selected enzymes are involved in synthesis and desaturation of fatty acids (*fatty acid synthase, FASN*; *stearoyl-CoA desaturase (delta 9 desaturase)*, *SCD*) and the two remaining enzymes are involved in fatty acids catabolism (*hormone sensitive lipase*, *LIPE*; *lipoprotein lipase*, *LPL*). *FASN* gene is located on Sus scrofa chromosome 12 (Muñoz et al., 2003), and the coded protein plays an essential role in long-chain fatty acid synthesis, starting from acetyl CoA and using malonyl-CoA as a 2 carbon donor and NADPH as reducing equivalent (Wakil, 1989; Menendez et al., 2009). In pigs both *SCD* and *LPL* genes are localised on chromosome 14 (Gu et al., 1992; Ren et al., 2003). SCD catalyses the desaturation of palmitoyl-CoA and stearoyl-CoA at the position Δ9 producing de novo palmitoleoyl-CoA and oleoyl-CoA, while LPL has the dual function of hydrolysing the circulating chylomicron triglycerides to diglycerides and of ligand/bridging factor for receptor mediated lipoprotein uptake. On the other hand, LIPE hydrolyses the triglycerides stored in muscle to diglycerides, then to monoglycerides and at the end to free fatty acids. *LIPE* gene has been assigned to porcine chromosome 6, and its position coincides with a Quantitative Trait Locus (QTL) region linked to sensory quality in porcine meat (Pena et al., 2013). In the present study, we analysed both the protein quantifications and the transcription profiles of these four genes in the *Semimembranosus* muscle of two groups of Italian Large White (ILW) pigs divergent for IMF deposition, with the aims: i) of testing whether the mRNA and enzyme levels of FASN, LIPE, SCD and LPL differed between two groups of pigs divergent for IMF (LOW IMF group *vs*. HIGH IMF group), ii) of identifying common trends in the expression levels of the four studied genes and proteins, suggesting coexpression links.

The focus both on gene and protein levels of the analysed enzymes could be useful to highlight a possible involvement of FASN, LIPE, SCD and LPL proteins on IMF deposition in pig meat.

2 Materials and methods

2.1 Protein expression study

2.1.1 Sampling

For the present study, a set of 155 pigs was selected among a population of 950 Italian Large White (ILW) pigs. As reported in Davoli et al. (2016), using the Soxhlet extraction method the whole sample of 950 ILW pigs has been characterised for IMF content, reported as percentage (grams of IMF on 100 grams of pig *Semimembranosus* muscle). The 155 individuals used for the quantitation of FASN, SCD, LIPE and LPL enzymes in *Semimembranosus* muscle have been selected among the 950 ILW pigs based on their extreme and divergent IMF phenotype (Table 1) and divided in two groups differing for IMF content (LOW IMF group and HIGH IMF group). The LOW IMF group consists of 77 pigs presenting IMF values lower than the average IMF level of the 950 pigs (total population) minus one standard deviation unit. Furthermore, the HIGH IMF group consists of individuals with IMF contents higher than the average IMF value of the total population plus 3.5 standard deviation units. The two IMF divergent groups have been chosen avoiding as much as possible full and half sibs, in order to prevent the family effect on protein and gene quantitation results. Additionally, since sex and batch may influence protein and gene expressions the two groups were balanced for these two factors. The pigs are pure breed animals included in the Italian sib test genetic evaluation scheme performed by ANAS (Associazione Nazionale Allevatori Suini, ANAS; www.anas.it), reared in the same environmental conditions at the genetic test station with a *quasi ad libitum* feeding level (60% of the pigs was able to ingest the entire supplied ration). The sib test program calculates the estimated genetic value of each candidate boar testing three of its full sibs, two females and one castrated male. For this reason, the considered sample of 155 individuals is composed

of two thirds of sows and one third of castrated males (Table 1), with the purpose of maintaining the proportions of the total population and of using a representative sample of the 950 pigs population. At the end of the test, the animals were transported to a commercial abattoir located about 25 km from the test station in accordance with Council Rule (EC) No. 1/2005 regarding the protection of animals during transport and related operations and, amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No. 1255/97. At the slaughterhouse, the pigs were electrically stunned and bled in a supine position in agreement with Council Regulation (EC) No. 1099/2009 regarding the protection of animals at the time of slaughter. All slaughter procedures were monitored by the veterinary team appointed by the Italian Ministry of Health. Moreover, for all the pigs, ANAS provided us with the Estimated Breeding Values (EBVs): Average Daily Gain (ADG, calculated from 30 to 155 kg of live weight with *quasi ad libitum* feeding level, expressed in grams), Backfat Thickness (BFT, recorded *post mortem* at the level of *Gluteus medius* muscle, expressed in mm), Lean Cuts (LC, the sum of neck and loin weight, expressed in kg), Feed Conversion Ratio (FCR, obtained from feed intake recorded daily and body weight measured bimonthly, expressed in units), and Ham Weight (HW, expressed in kg). The listed EBVs have been calculated by ANAS according to the statistical model reported by Russo et al. (2000). Moreover, the slaughterhouse technicians provided us with the phenotypic measures of the carcass weight expressed in kg (carcass weight), the percentage of lean cuts over the total carcass weight (%lean), and the backfat and loin thickness measured at 8 cm off the midline of the carcass at the level placed between the third and fourth last ribs measured with Fat-O-Meter (FOM).

2.1.2 Protein extraction and total protein quantitation

At the slaughterhouse, *Semimembranosus* muscle tissue was quickly frozen in Liquid Nitrogen (LN2) and then stored at -80°C for subsequent total protein extraction. Approximately 300 mg of tissue sample were homogenized on ice in 900 µl of T-PER Tissue Protein Extraction (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), to which 1x protease and phosphatase inhibitors (PhosSTOP™, Roche, Hoffmann-La Roche, Basel,

Switzerland) were added. Tissue lysates were then centrifuged at 15,000 x g for 20 min at 4°C to remove lipids and insoluble debris. For LPL, before the quantitation of the total proteins and the quantification of LPL enzyme, the extracted proteins were concentrated through a filtering step, using Amicon® Ultra-4 filters (Merck Millipore, Darmstadt, Germany). This additional step was needed to obtain LPL quantitation within the sensitivity range of the commercial ELISA kit used for the quantitation of this protein.

The total extracted proteins were quantified using the BCA reagent (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and the optical density of each sample was determined using a microplate optical reader.

2.1.3 Quantification of intramuscular FASN, LIPE, SCD and LPL enzymes

FASN, LIPE, SCD and LPL were quantitatively detected using ELISA kits produced by Cusabio (Cusabio Biotech, Wuhan, China), according to manufacturer's instructions. Briefly, 100 µl of blank, standards or samples were added to well, covered, incubated for 2 hours at 37°C and the liquid of each well was removed without washing. Then 100 µl of biotin antibody working solution was added to each well, incubated for 1 hour at 37°C and then washed three times with wash buffer (200 µl). At each well, 100 µl of HRP avidin working solution were added, the well covered, incubated for 1 hour at 37°C and washed three times with wash buffer (200 µl). Finally, TMB substrate (90 µl) was then added to each well, covered, dark incubated for 30 minutes at 37°C and stopped with 50 µl of stop solution. The optical density of each well was determined within 5 minutes using a microplate reader at 450 nm.

For all the samples, the relative quantification of each enzyme was calculated, through the ratio between the enzyme absolute quantitation and the total extracted protein amount.

2.2 Gene expression study

2.2.1 Sampling

The gene expression study was carried out on a smaller sample, composed of 47 ILW pigs chosen among the overall group of 155 animals. These selected pigs, which were extreme and divergent for IMF phenotype (Table 1), were the progeny of 36 boars and 44 sows. Both Subset 1 (for the low IMF pigs) and Subset 2 (for the high IMF individuals) have been chosen avoiding as much as possible full and half sibs.

2.2.2 RNA source, total RNA extraction and cDNA preparation

Semimembranosus muscle samples were withdrawn at the slaughterhouse and immediately frozen at -80°C. Total RNA was then extracted using TRIZOL reagent (Invitrogen Corporation, Carlsbad, California), the quality and integrity of the RNAs were both checked and RNA was retrotranscribed to cDNA as described in Davoli et al. (2011).

2.2.3 Gene expression quantitation

The quantitative Real-Time PCR (qRT-PCR) standard curve method (Pfaffl, 2004) was used to analyse the four genes expression. qRT-PCR was performed on Rotor Gene™ 6000 (Corbett Life Science, Concorde, New South Wales) using 5 µl of SYBR® Premix Ex Taq™ (TAKARA Bio INC, Olsu, Shiga, Japan), 5 pmol of each primer, 2 µl of cDNA template diluted 1:10 and make up to the total volume of 10 µl with water. Rotor Gene™ 6000 protocol was optimised using specific annealing temperatures for each primer couple (Supplementary Table 1). Six genes were tested to be used as normalising genes (*polymerase (RNA) II (DNA directed) polypeptide A, 220kDa*, *POLR2A; beta-2-microglobulin, B2M; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta, YWHAZ; hypoxanthine phosphoribosyltransferase 1, HPRT1; TATA box binding protein, TBP; peptidylprolyl isomerase A (cyclophilin A), PPIA*) and using NormFinder (Andersen et al., 2004) were identified the three most stable ones to be used as normalising genes (*B2M*, *YWHAZ*, and *HPRT1*). For the normalising and the 4 studied genes a specific standard curve was obtained, amplifying 11 serial dilutions (from 10^9 to 50 molecules/µl) of a known concentration sample of cDNA amplicon, obtained with a PCR with the external primer pairs (Supplementary Table 1). The PCR efficiency was calculated as E = 10 exp (-1/slope), with a

range between -2.7 and -4.3, indicating a good PCR efficiency results. All the PCR products were checked on a polyacrylamide gel and washed from primer dimers with the QIAquick® PCR Purification Kit (QIAGEN, Venlo, Netherlands). The absence of unspecific amplicons during qRT-PCR on Rotor Gene™ 6000 was tested using the melt step after the cycling. The variation coefficient (CV = Standard Deviation of the Crossing Points/Average of the Crossing Points) of the replicated analysis for each samples (three in two different qRT PCRs) was accepted for CV < 0.2.

2.3 Statistical analysis

All the statistical analyses were carried out using SAS software, version 9.4 (SAS Inst. Inc., Cary, NC). To decide what statistical approach to use, we performed a general linear model (GLM) where we tested the fixed effects of sex, batch and group (LOW and HIGH IMF groups) on the studied gene and protein levels. As sex and batch did not affect the gene and protein levels, we decided to take into account only the group effect, comparing the protein and gene levels between the divergent groups of pigs. PROC NPAR1WAY command of SAS (the Wilcoxon test) was used to compare the protein levels between the groups of pigs divergent for IMF deposition (as protein values were not normally distributed), and the PROC TTEST of SAS (Student's t test) was utilised to compare the gene expression data between groups (as gene expression values were normally distributed). The PROC CORR SAS command was considered to calculate the correlations between the different gene and protein expressions.

3 Results

The main results are reported in Figure 1, Figure 2 and in Table 2, where for the two IMF divergent groups the protein and gene expression quantifications are showed. In particular we investigated both the mRNA and protein levels of *FASN*, *LIPE*, *SCD* and *LPL* genes, which have been already described in literature to be likely involved in IMF deposition. However, to date, the association of these genes with IMF deposition was reported mainly on the basis of their mRNA levels while in the present research both the mRNA and protein

amounts were considered. For the quantification of these four enzymes in the two groups of ILW pigs extreme for IMF, we used commercially available ELISA kits. Owing to some problems in SCD and LPL protein quantitation and in order to avoid uncertain data, we decided to present and compare only the most reliable quantification values, maintaining however the divergence between the two groups for IMF value and balancing the samples for sex (Supplementary Table 2).

The means ± the standard errors of the quantified enzymes in the LOW IMF and HIGH IMF groups are represented in Figure 1. It is worth noting that LIPE is the only enzyme showing a higher amount in samples with LOW IMF deposition ($P<0.05$) (Figure 1 C), while SCD protein presents a trend ($P<0.1$) and on average a higher enzyme expression in the HIGH IMF group (Figure 1 A). The transcription levels obtained for the four considered genes are reported in Figure 2: the two groups (LOW and HIGH IMF) show differences in the mRNA levels for *FASN*, *LPL* and *SCD* genes. In Table 2 are indicated the means ± the standard deviations of the gene and enzyme expressions in the LOW and HIGH IMF groups, in the LOW and HIGH IMF sows and castrated males, and in the two sexes. In the same table are reported the significant comparisons between groups. In order to identify links between gene transcription levels and enzyme expressions, the correlations between the mRNA and the protein levels were performed, and the results reported in Table 3. The lack of correlation between the mRNA level of each gene and the expression of its coded protein clearly stands out. Furthermore, we calculated the correlations between each one of the analysed mRNA and protein expressions and all the other available quantitation data, in order to look for the presence of some possible coexpressions (Table 3). This approach revealed the presence of two significant correlations between the mRNA of *LIPE* gene and the amount of LPL and SCD proteins. Furthermore, *FASN* gene resulted highly correlated to *SCD* and *LPL* gene expressions, while for the enzymes a single positive correlation between LIPE and FASN was identified (Table 3). In particular, this positive and high correlation between LIPE and FASN enzymes showed to be strongly linked to sex, as only sows presented a highly significant correlation coefficient, not observed in castrated males (Table 4). Furthermore, in

order to find some possible links between gene and protein expressions and carcass traits, a correlation analysis considering gene and enzymes expression levels towards EBVs and phenotypic measures of carcass traits was carried out (Supplementary Table 3). The results in Supplementary Table 3 show low correlations between the gene and protein expression data and the EBVs or phenotypic measures, but all the associations we found are consistent with the roles of the studied genes and enzymes. For example, FASN is negatively correlated to ADG and HW EBVs, and to carcass weight measure, in agreement with the lipogenic role of this enzyme. Moreover, IMF content resulted to be positively associated with *FASN*, *SCD* and *LPL* gene expression levels, and among the tested enzymes only LIPE showed a negative correlation with IMF deposition (Supplementary Table 3).

4 Discussion

The primary aim of the study was to investigate the expression levels of FASN, LIPE, SCD and LPL enzymes between two groups of ILW pigs divergent for IMF deposition, in order to look for the involvement of these proteins in porcine IMF deposition. The results suggest a prominent role of LIPE enzyme in IMF hydrolysis: indeed, low IMF pigs have a significantly higher amount of LIPE enzyme as compared to the high IMF group (Figure 1 C and Table 2). This result agrees with the role of LIPE, which hydrolyses to free fatty acids the triglycerides stored in adipose tissue. According to this result, we also found a negative correlation between IMF deposition and LIPE enzyme amount (Supplementary Table 3). The different LIPE relative quantitation between high and low IMF individuals is also maintained between IMF divergent pigs of the same sex, indicating the importance of LIPE activity in IMF deposition for both castrated male and female individuals (Table 2). To date, in different animal species several authors have found associations between *LIPE* gene or enzyme expressions and the carcass traits (Zhao et al., 2010 in pigs; Ying et al., 2013 in mice; Zhang et al., 2014 in yaks). In particular, Zhao et al. (2009) found a divergent expression of *LIPE* gene and protein between 12 Wujin pigs with high IMF deposition and 12 Landrace pigs with low IMF deposition. Due to the different diet compositions and the use of two samples

coming from distinct pig breeds, the diverging *LIPE* mRNA levels found by the authors between the two breeds may be affected by the different characteristics of the diets. However, on the basis of the results of the present study, it is possible to suggest a diet independent role of LIPE on IMF deposition, as LIPE enzyme showed different expressions between IMF divergent pigs, although they were reared in the same environmental conditions.

On the other hand, in pigs with a more pronounced IMF deposition a higher level of SCD protein was found, but the difference between the SCD enzyme expressions in LOW IMF and HIGH IMF groups showed only a trend towards significance ($P < 0.1$) (Figure 1). This result suggests that SCD enzyme could have an important role in IMF metabolism and is in agreement with the data reported by Wu et al. (2013), who detected an overexpression of *SCD* gene in high IMF Jinhua pigs as compared to Landrace pigs.

In addition, we tested also the linear correlation between the genes and the relative protein expressions (Table 3), finding that no one of the enzyme amounts significantly covaried with the relative gene transcription level. This result indicates that post-transcriptional regulation for these genes may play an essential role in the modulation of their enzyme synthesis and activity. However, despite this result, it is worth noting that *FASN*, *LPL* and *SCD* maintained on average a higher expression of both mRNA and protein levels in high IMF pigs as compared to the low IMF group (Figure 1 and 2) suggesting the existence of a conserved trend between the transcription and translation levels of these three genes.

Furthermore, the strong correlation found between LIPE and FASN (Table 3) was further investigated through an additional correlation analysis performed in castrated males and in sows (Table 4). Based on the results obtained, in muscle tissue we can suppose the activation of distinct biological pathways controlling IMF adipogenesis between female and male individuals. Mukherjee et al. (2014) found that in rat white adipose tissue the quantity of LIPE enzyme varies together with the expression of Caveolin1 (CAV1): in particular, CAV1 showed to be stimulated by estrogen (the primary female sex hormone) and suppressed by

androgen (typically the male sex hormone). On the other side, CAV1 is coexpressed with FASN and interacts with this enzyme in human prostate cancer (Di Vizio et al., 2008). CAV1 is an important target in sex hormone dependent regulation of several metabolic pathways, particularly in cancer and diabetes (Mukherjee et al., 2014). Considering the results reported in literature, it is possible to hypothesise that the strong correlation we found in sows between LIPE and FASN proteins may be the effect of an estrogen activated pathway, possibly linking these two enzymes through the mediation of CAV1. Anyway, further studies are needed to prove in pigs this hypothesis.

In Table 3, also the correlation between *FASN* and *SCD* gene expressions stands out. The expressions of these two genes have already been found to covary in *Longissimus dorsi* muscle between pigs with divergent muscle lipid deposition (Wang et al., 2015). Nevertheless this coexpression was not detected at the protein level (Table 3).

Moreover, the correlations found between the EBVs or carcass traits and the gene and protein expression levels agree with the known roles of the studied genes: FASN and SCD are both enzymes involved in the synthesis and desaturation of fatty acids, and in fact showed negative correlations with the EBV of Average Daily Gain and more generally to lean mass deposition, causing a worsening in Feed Conversion Ratio EBV (Supplementary Table 3).

5 Conclusions

The study of FASN, LIPE, LPL and SCD gene and protein expression levels allowed obtaining a more comprehensive view of their involvement in pig intramuscular fat deposition. The results suggest for LIPE and SCD enzyme a role in muscle fat deposition and indicate that these two genes may be involved in similar regulatory pathways and respond to similar transcription cues. Anyway, future studies are needed to better understand the role of *LIPE* gene and its coded protein in pork quality. Finally, in female individuals a poorly known correlation between LIPE and FASN enzymes was found, suggesting the need of dedicated studies aimed at identifying and elucidating the pathways involved.

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412

Figure captions

Figure 1. Relative quantitation of the studied enzymes in the LOW and HIGH IMF groups. Mean values are shown and error bars represent the standard errors.

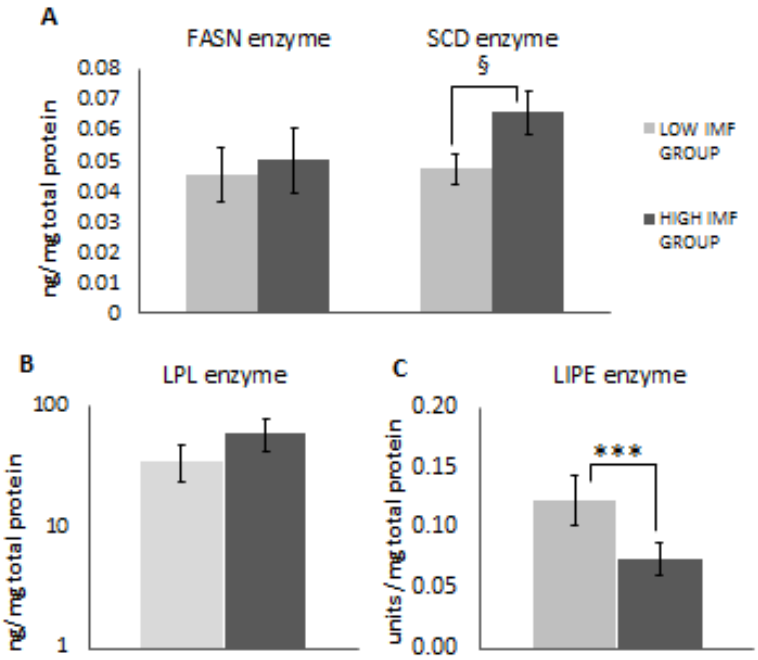
Section A: relative quantitation of FASN and SCD enzymes in the low IMF and high IMF groups; section B: relative quantitation of LPL enzyme in the low IMF and high IMF groups; section C: relative quantitation of LPL enzyme in the low IMF and high IMF groups.

Significant differences between the two divergent groups are expressed with *** for $P<0.001$. [§] is used to indicate differences with P value<0.10.

Figure 2. Relative quantitation of the gene expressions in Subset 1 (LOW IMF) and Subset 2 (HIGH IMF). Mean values are shown and error bars represent the standard errors.

Significant differences between the two divergent groups are expressed with * for $P<0.05$ and ** for $P<0.01$. [§] is used to indicate differences with P value<0.1.

Figure 1. Relative quantitation of the studied enzymes in the LOW and HIGH IMF groups. Mean values are shown and error bars represent the standard errors.

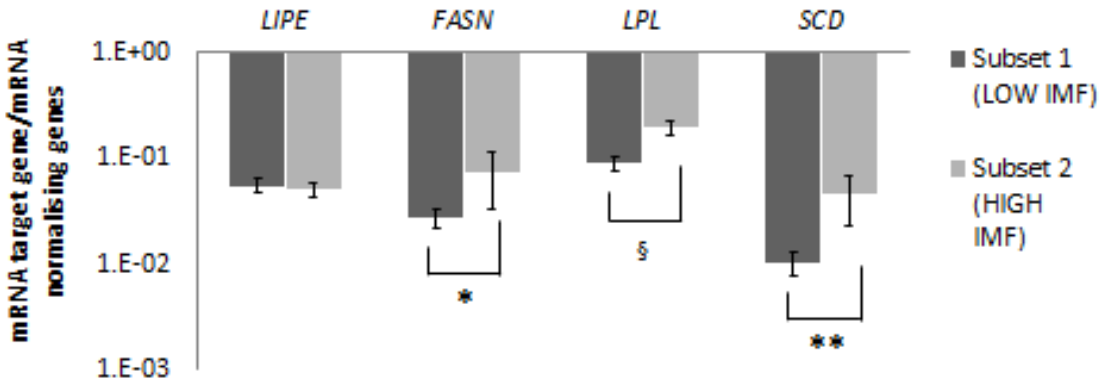


Section A: relative quantitation of FASN and SCD enzymes in the low IMF and high IMF groups; section B: relative quantitation of LPL enzyme in the low IMF and high IMF groups; section C: relative quantitation of LPL enzyme in the low IMF and high IMF groups.

The significant comparison between the two divergent groups is expressed with *** for $P<0.001$. § is used to indicate differences with P value<0.10.

436 **Figure 2.** Relative quantitation of the gene expressions in Subset 1 (LOW IMF) and Subset 2

437 (HIGH IMF). Mean values are shown and error bars represent the standard errors.



438

439 The significant comparisons between the two divergent groups are expressed with * for

440 $P<0.05$ and ** for $P<0.01$. § is used to indicate differences with P value<0.10.