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Effect of polymorphisms in the *FASN*, *OLR1*, *PPARGC1A*, *PRL* and *STAT5A* genes on bovine milk-fat composition

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Summary

The aim of our study was to estimate effects of polymorphisms in the *ATP-binding cassette G2* (*ABCG2*), *fatty acid synthase* (*FASN*), *oxidized low-density lipoprotein receptor 1* (*OLR1*), *peroxysome proliferator-activated receptor-γ coactivator-1α* (*PPARGC1A*), *prolactin* (*PRL*) and *signal transducer and activator of transcription 5A* (*STAT5A*) genes on milk production traits and detailed milk-fat composition. Milk-fat composition phenotypes were available for 1905 Dutch Holstein–Friesian cows. First, the presence of each SNP in the Dutch Holstein–Friesian population was evaluated by direct sequencing of the PCR product surrounding the SNP in 22 proven Dutch Holstein–Friesian bulls. The *ABCG2* SNP did not segregate in the bull population. Second, we genotyped the cows for the $FASN_{g.16024G>A}$, $FASN_{g.17924A>G}$, $OLR1_{g.8232C>A}$, $PPARGC1A_{c.1790+514G>A}$, $PPARGC1A_{c.1892+19G>A}$, $PRL_{g.8398G>A}$ and $STAT5A_{g.9501G>A}$ polymorphisms, and estimated genotype effects on milk production traits and milk-fat composition. $FASN_{g.17924A>G}$ and $OLR_{g.8232C>A}$ had a significant effect ($P < 0.05$) on milk-fat percentage. However, we were not able to confirm results reported in the literature that showed effects of all evaluated polymorphisms on milk-fat percentage or milk-fat yield. All polymorphisms showed significant effects ($P < 0.05$) on milk-fat composition. The polymorphisms in *FASN* and *STAT5A*, which had an effect on C14:0 and were located on chromosome 19, could not fully explain the quantitative trait locus for C14:0 that was previously detected on chromosome 19 in a genome-wide scan using linkage analysis.

Keywords dairy cattle, *FASN*, fatty acid, *OLR1*, *PPARGC1A*, *PRL*, single nucleotide polymorphism, *STAT5A*.

Introduction

Recent studies have revealed large genetic variation in bovine milk-fat composition (Soyeurt *et al.* 2007; Stoop *et al.* 2008). Candidate genes underlying this variation may be found in fat synthesis and metabolism pathways, which are under the control of multiple genes. Information on effects of DNA polymorphisms on milk-fat composition is scarce, because milk-fat composition data are, unlike milk-fat percentage and milk-fat yield, not routinely collected in milk recording schemes. Polymorphisms in the *diacylglycerol acyltransferase 1* (*DGAT1*) and *stearoyl-CoA desaturase 1* (*SCD1*) genes have been shown to affect the composition of bovine milk-fat in different cattle populations (Mele *et al.* 2007; Moioli *et al.* 2007; Schennink *et al.* 2007, 2008). Single nucleotide polymorphisms (SNPs) in several other genes that play a role in fat synthesis or metabolism pathways have been associated with milk-fat percentage or milk-fat yield. In the current study, we evaluated associations between SNPs in those genes and milk production and milk-fat composition traits. The genes under study were *ATP-binding cassette G2* (*ABCG2*), *fatty acid synthase* (*FASN*), *oxidized low-density lipoprotein receptor 1* (*OLR1*), *peroxysome proliferator-activated receptor-γ coactivator-1α* (*PPARGC1A*), *prolactin* (*PRL*) and *signal transducer and activator of transcription 5A* (*STAT5A*). Previous studies in different cattle populations have reported effects of SNPs in these genes on the routinely collected traits milk-fat percentage, milk-fat yield or both (Dybus 2002; Brym *et al.* 2004, 2005; Cohen-Zinder *et al.* 2005; Dybus *et al.* 2005; Weikard *et al.* 2005; Khatib *et al.* 2006; Roy *et al.* 2006; Morris *et al.* 2007).

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The first objective of this study was to replicate the association analysis between polymorphisms in the *ABCG2*, *FASN*, *OLR1*, *PPARGC1A*, *PRL* and *STAT5A* genes, and milk production traits. Reproduction of the reported positive associations in an independent population is important to allow evaluation of the candidate gene studies. The second objective of this study was to estimate effects of the above-mentioned polymorphisms on detailed milk-fat composition. With the exception of *FASN*, no information is available on the effects of the genes under study on milk-fat composition. Milk-fat composition is important for the nutritional value (with regard to human health) and the technological properties (e.g. the spreadability of butter) of milk-fat.

Materials and methods

Animals

This study is part of the Dutch Milk Genomics Initiative, which focuses on the genetic background of detailed milk composition. Phenotypic data were available from 1905 cows from 398 commercial herds in the Netherlands. Cows descended from one of five proven bulls (871 cows), from 1 of 50 young bulls (844 cows) or from other proven bulls (190 cows). The last group was added to ensure that at least three cows were sampled per herd. The breeding organization CRV (Arnhem, the Netherlands) provided pedigrees of the cows, which were over 87.5% Holstein–Friesian (HF). Each cow was between day 63 and 282 of first lactation, and milked twice a day. Blood or semen samples for DNA analysis were collected from the cows, the five proven and 50 young bulls. To test SNP frequencies in a small group of animals representative of the Dutch HF cattle population, semen was collected from 17 other frequently used proven HF bulls.

Phenotypes

One morning milk sample of 500 ml per cow was collected between February and March 2005, which is the winter period. Milk-fat composition was measured by gas chromatography at the COKZ laboratory (Netherlands Controlling Authority for Milk and Milk Products, Leusden, the Netherlands) as described by Schennink *et al.* (2007). The fatty acids were expressed as weight-proportion of total fat weight. Fat and protein percentages were measured by infrared spectroscopy, using a MilkoScan FT6000 (Foss Electric) at the Milk Control Station (Zutphen, the Netherlands). Fat and protein yields were calculated by multiplying each percentage by the morning milk yield. Yield data were missing for 138 cows. In this study, 28 traits were analysed: fat and protein percentage, fat, protein and milk yield, the individual fatty acids C6:0, C8:0, C10:0, C10:1, C12, C12:1, C14:0, C14:1*cis*9, C16:0, C16:1*cis*9, C18:0, C18:1*cis*9, C18:2*cis*9,12, C18:2*cis*9,*trans*11 (CLA), C18:3*cis*9,12,15, the group C18:1*trans* fatty acids, and the unsaturation indices for C10, C12, C14, C16 and C18, CLA and total fatty acids. Unsaturation indices were calculated by expressing each product as a proportion of the product plus substrate, multiplied by 100 (Kelsey *et al.* 2003), e.g.

$$\text{C18index} = \frac{\text{C18} : 1cis9}{\text{C18} : 1cis9 + \text{C18} : 0} * 100.$$

The overall means, standard deviations and heritabilities (as calculated by Schennink *et al.* 2009 and Stoop *et al.* 2009) of the traits analysed in this study are shown in Table S1.

Genotypes

Genomic DNA was isolated from blood samples using Puregene (Gentra; Qiagen) and from semen samples according to Ganai *et al.* (2009). The presence of the SNP in the Dutch HF population was evaluated by direct sequencing of the PCR product surrounding the SNP in the 22 proven bulls: the five proven bulls that have daughters in the Milk Genomics Initiative population, and 17 other frequently used proven bulls. Primers were designed (Table S2) based on GenBank sequences AJ871176 (*ABCG2*), AF285607 (*FASN*), NW_215807 (*OLR1*), AY321517 and NC_007304 (*PPARGC1A*), AF426315 (*PRL*) and AJ237937 (*STAT5A*). The $ABCG2_{\text{g.62569A>C}}$ SNP did not segregate in either the 22 proven bulls or the 50 young bulls (the A allele was fixed), and therefore excluded from further analysis. Cows were genotyped by one of three methods described below. Genotypes for $FASN_{\text{g.16024A>G}}$, $OLR1_{\text{g.8232A>C}}$, $PPARGC1A_{\text{c.1790+514G>A}}$, $PPARGC1A_{\text{c.1892+19C>T}}$ and $PRL_{\text{g.8398A>G}}$ were assayed by the SNaPshot single base primer extension method (Applied Biosystems) as previously described (Schennink *et al.* 2008). Genotyping primers are shown in Table S2. The $STAT5A_{\text{g.9501A>G}}$ polymorphism was genotyped using a GoldenGate assay (Illumina) according to the manufacturer's protocol. The $FASN_{\text{g.17924A>G}}$ polymorphism was genotyped by a custom Infinium assay (Illumina) according to the manufacturer's protocol.

Linkage disequilibrium

HAPLOVIEW software (Barrett *et al.* 2005) was used to calculate linkage disequilibrium (r^2) between the SNPs that were located on the same chromosomes: the two SNPs in *FASN* and $STAT5A_{\text{g.9501G>A}}$, located on BTA19 and the two SNPs in *PPARGC1A*, located on BTA6 (Hill & Robertson 1968). Calculations in HAPLOVIEW are based on genotypic data without considering the pedigree structure.

Statistical analysis

Phenotypes of all 1905 animals were used to estimate variance components and systematic environmental effects

as described in Schennink *et al.* (2007). Genotypes were not available for all 1905 animals and this caused problems in adjusting for herd effects, because some herds had less than three observations. Therefore we used pre-corrected data to estimate genotype effects. Data were pre-corrected for days in milk, age at calving, season of calving and herd. Genotype effects were estimated using the following Animal Model in ASReml, with the variance of additive genetic effects at fixed values (Gilmour *et al.* 2002):

$$y_{ij} = \mu + \mathrm{SNP}_i + a_j + e_{ij},$$

where y_{ij} is the dependent variable adjusted for systematic environmental effects of days in milk, age at calving, season of calving and herd; SNP_i is the effect of SNP genotype i; a_j is the random additive genetic effect of animal j; e_{ij} is the random residual effect. Additive genetic relations between animals were accounted for.

In a previous study, we performed a genome-wide scan to detect quantitative trait loci (QTL) for bovine milk-fat composition. This genome-wide scan consisted of 849 cows representing five large and two small paternal half-sib families in a weighted across-family regression. Significant QTL were detected on BTA6, 14, 15, 16, 19 and 26 (Schennink *et al.* 2009; Stoop *et al.* 2009). Both *FASN* and *STAT5A* are located on BTA19, and to investigate whether the SNPs in *FASN* or *STAT5A* contributed to the QTL detected on BTA19 for C14:0, the QTL analysis was repeated using phenotypes corrected for these SNP effects in *FASN* or *STAT5A* (see Schennink *et al.* 2009 and Stoop *et al.* 2009, for details).

Results and discussion

In this study, SNPs in *FASN*, *OLR1*, *PPARGC1A*, *PRL* and *STAT5A* loci were genotyped in Dutch HF cattle, and their effects on milk production traits and detailed milk-fat composition were estimated. Detailed information on the SNPs and the population where they have been reported can be found in Table S3. Allele frequencies are shown in Table 1. All genotypes, except for $PPARGC1A_{\mathrm{c.1892+19C>T}}$, were in Hardy–Weinberg equilibrium. As the sampled population is not a random sample, but a selection of a number of families, there is no reason to exclude $PPARGC1A_{\mathrm{c.1892+19C>T}}$ from further analyses. Effects of the genotyped polymorphisms on milk production traits and milk-fat composition are shown in Table 2. Only effects with a *P*-value smaller than 5% are listed in Table 2. Results will be discussed for each gene.

Table 1 Allele frequencies of the genotyped polymorphisms.

Gene locus	BTA	SNP[1]	Number of genotyped animals[2]	Allele frequency[3]
			Bulls	
ABCG2	6	g.62569A>C	72	1.00
			Cows	
FASN	19	g.16024G>A	1724	0.89
		g.17924A>G	1688	0.53
OLR1	5	g.8232C>A	1724	0.71
PPARGC1A	6	c.1790+514G>A	1724	0.80
		c.1892+19C>T	1722	0.75
PRL	23	g.8398G>A	1722	0.80
STAT5A	19	g.9501G>A	815[4]	0.57

[1]SNP location is numbered according to the GenBank sequence in case of *FASN* (AF285607), *PRL* (AF426315), *STAT5A* (AJ237937) and *ABCG2* (AJ871176), and according to reference in case of *OLR1* (Khatib et al. 2006), and *PPARGC1A* (Weikard et al. 2005).
[2]Number of genotyped animals indicates how many of the 1905 cows were genotyped for the polymorphism. Genotypes were missing for some animals because either no DNA sample was available or because the polymorphism could not be genotyped unambiguously. The $ABCG2_{\mathrm{g.62569A>C}}$ SNP was sequenced in 22 proven and 50 young bulls, but did not segregate in the bull population.
[3]The allele frequency of the first allele is given, e.g. the frequency of $ABCG2_{\mathrm{g.62569A}}$ is 1.
[4]For the $STAT5A_{\mathrm{g.9501G>A}}$ SNP only the daughters of the five proven bulls and two young bulls were genotyped.

ABCG2

The multidrug transporter encoded by *ABCG2* is shown to be strongly induced in the mammary glands of mice, cows and humans during lactation, and is responsible for the secretion of clinically and toxicologically important substrates into mouse milk (Jonker *et al.* 2005). Cohen-Zinder *et al.* (2005) proposed a non-synonymous mutation in *ABCG2*, leading to a Tyr>Ser amino acid change, as the causative polymorphism underlying a QTL on *Bos taurus* autosome (BTA) 6 affecting milk yield, milk-fat percentage and milk-protein percentage. The C allele of the $ABCG2_{\mathrm{g.62569A>C}}$ SNP (or p.Tyr581Ser) did not segregate in our bull population (22 proven and 50 young bulls). This result is in line with results from Ron *et al.* (2006), who investigated the allele frequencies of this SNP in 35 cattle breeds, including Israeli, German and US Holstein, and found the A allele to be predominant in all populations, with frequencies ranging from 0.80 to 1. The *ABCG2* A allele, which decreases milk yield and increases protein and fat percentage (Cohen-Zinder *et al.* 2005), is economically favourable for most selection indexes, which might explain high frequency of this allele.

FASN

Fatty acid synthase is a multifunctional enzyme complex that catalyses *de novo* fatty acid synthesis and has been put forward as a candidate gene for milk-fat percentage as well as fat composition of milk and beef. SNPs in different exons of the *FASN* gene, and different functional domains of the

Table 2 Effects (with SE) of the polymorphisms in *FASN*, *OLR1*, *PPARGC1A*, *PRL* and *STAT5A* on milk-fat composition.

$FASN_{g.16024G>A}$	GG (n = 1350)	GA (n = 358)	AA (n = 16)	P-value
C14:0	0	−0.21 (0.05)	−0.38 (0.19)	**
C18:2*cis*9,12	0	0.02 (0.01)	0.08 (0.04)	
$FASN_{g.17924A>G}$	AA (n = 465)	AG (n = 849)	GG (n = 374)	
Fat percentage	0	−0.07 (0.04)	−0.13 (0.05)	
C14:0	0	−0.14 (0.04)	−0.23 (0.06)	**
C18:1*cis*9	0	0.10 (0.09)	0.36 (0.12)	
Total index	0	0.11 (0.10)	0.36 (0.13)	
$OLR1_{g.8232C>A}$	CC (n = 847)	CA (n = 744)	AA (n = 133)	
Fat percentage	0	−0.03 (0.03)	−0.16 (0.06)	
C18:0	0	−0.08 (0.06)	−0.29 (0.11)	
C18 index	0	0.31 (0.18)	0.89 (0.33)	
CLA index	0	0.27 (0.19)	0.94 (0.34)	
$PPARGC1A_{c.1790+514G>A}$	GG (n = 1084)	GA (n = 574)	AA (n = 66)	
C16:1*cis*9	0	0.05 (0.02)	0.10 (0.04)	*
C16 index	0	0.10 (0.04)	0.21 (0.10)	
$PPARGC1A_{c.1892+19C>T}$	CC (n = 937)	CT (n = 692)	TT (n = 93)	
C14:1*cis*9	0	−0.04 (0.01)	−0.04 (0.03)	*
C12 index	0	−0.09 (0.03)	−0.07 (0.06)	*
C14 index	0	−0.31 (0.09)	−0.22 (0.19)	*
C18 index	0	−0.39 (0.19)	−0.81 (0.40)	
$PRL_{g.8398G>A}$	CC (n = 1097)	CT (n = 560)	TT (n = 65)	
C10:0	0	−0.04 (0.02)	−0.07 (0.04)	
C12:1	0	-3.3×10^{-3} (0.1×10^{-3})	-3.3×10^{-3} (3.0×10^{-3})	
$STAT5A_{g.9501G>A}$	GG (n = 267)	GA (n = 400)	AA (n = 148)	
C10:0	0	0.03 (0.03)	0.11 (0.04)	
C14:0	0	0.06 (0.06)	0.32 (0.09)	**
C14:1	0	−0.01 (0.02)	−0.07 (0.03)	
C16:1*cis*9	0	0.2×10^{-3} (0.02)	−0.09 (0.03)	*
C10 index	0	−0.09 (0.14)	−0.55 (0.20)	
C12 index	0	−0.03 (0.04)	−0.19 (0.06)	*
C14 index	0	−0.12 (0.13)	−0.73 (0.19)	**
C16 index	0	0.04 (0.06)	−0.18 (0.09)	*
C18 index	0	−0.17 (0.28)	−0.95 (0.40)	

All listed effects have a P-value < 0.05; *P < 0.01; **P < 0.001.

FASN protein, have been associated with milk-fat percentage (Roy *et al.* 2006) and with medium and long chain fatty acid content of milk (Morris *et al.* 2007) and beef (Zhang *et al.* 2008). $FASN_{g.16024G>A}$ is a non-synonymous SNP leading to a Ala>Tyr amino acid change. The G allele frequency of 0.89 is the same as found by Roy *et al.* (2006), who named this SNP $FASN_{g.16009A>G}$. Morris *et al.* (2007) identified five SNPs in *FASN*, which included $FASN_{g.17924A>G}$, a non-synonymous SNP leading to a p.Tyr>Ala amino acid change, but not $FASN_{g.16024G>A}$. The $FASN_{g.17924A>G}$ A allele frequency of 0.53 in our population is higher than the frequencies found by Morris *et al.* (2007) in Friesian and Jersey cattle (0.31 and 0.13 respectively), but lower than the frequency of 0.62 reported in Angus beef cattle (Zhang *et al.* 2008). $FASN_{g.17924A>G}$ showed significant effects on milk-fat percentage in our cattle population (Table 3). No association of $FASN_{g.17924A>G}$ with milk-fat percentage is reported in the literature, however, an association of the other SNP in *FASN* ($FASN_{g.16024G>A}$), with milk-fat percentage has been reported (Roy *et al.* 2006).

Both SNPs in *FASN* affected C14:0, whereas $FASN_{g.16024G>A}$ also affected C18:2*cis*9,12, and $FASN_{g.17924A>G}$ also affected C18:1*cis*9 and the total index. Effects of SNPs in

Table 3 *FASN* and *STAT5A* genotypes of the seven sires from which daughters were included in the genome-wide scan for milk-fat composition[1], and QTL substitution effects for C14:0.

Sire	$FASN_{g.16024G>A}$	$FASN_{g.17924A>G}$	$STAT5A_{g.9501G>A}$	No. daughters in genome-wide scan	QTL allele substitution effect[1]	Segregating significantly[1]
1	GG	AA	GG	193	$0.09_{0.10}$	
2	GG	AA	AA	179	$0.72_{0.22}$	*
3	GG	AA	GG	170	$-0.20_{0.11}$	
4	GG	GG	AG	166	$-0.28_{0.11}$	*
5	GG	AG	AG	91	$0.08_{0.15}$	
6	GG	AG	AG	29	$-0.32_{0.30}$	
7	GG	AG	GG	21	$1.14_{0.33}$	*

[1]From Stoop et al. (2009).

FASN on milk-fat composition have been shown in a New Zealand study (Morris *et al.* 2007). In a milk-fat composition trial using Friesian sires with many daughters, Morris *et al.* (2007) identified one segregating sire that was significant for *FASN*. In another milk-fat composition trial using random Friesian and Jersey cows, they did not find an effect of the $FASN_{g.17924A>G}$ SNP. However, they did show association of two other *FASN* SNPs (a synonymous SNP in exon 32 and a SNP in intron 32) with C14:0 and C18:1*cis*9, but only in Jersey cattle and not in Friesian cattle. Association of the $FASN_{g.17924A>G}$ G allele with higher C14:0 and lower C18:1*cis*9 was also reported in beef cattle (Zhang *et al.* 2008). Zhang *et al.* (2008) showed that the resulting amino acid replacement (Thr>Ala) was located a few amino acids C-terminal to a candidate substrate-binding site. This binding site, and consequently the activity of the enzyme, may be influenced by the polymorphism.

OLR1

OLR1 is involved in fatty acid transport and binds and degrades the oxidized form of low-density lipoprotein. Khatib *et al.* (2006) identified *OLR1* as a functional and positional candidate gene for milk-fat percentage and milk-fat yield, and showed association of a SNP in the 3′-UTR of *OLR1* ($OLR1_{g.8232C>A}$) with milk-fat percentage and milk-fat yield in a population of North American Holstein cattle. The allele frequency of $OLR1_{g.8232C>A}$ is in line with the previous reports in Holstein cattle (Khatib *et al.* 2006, 2007a). Association of the allele of $OLR1_{g.8232C>A}$ with lower milk-fat percentage is in concordance with Khatib *et al.* (2006), although an association with lower milk-fat yield was not shown in this study.

$OLR1_{g.8232C>A}$ showed association with the long chain fatty acid C18:0, and with C18 and CLA indices. Long chain milk fatty acids originate from blood lipids, whereas short and medium chain milk fatty acids originate from *de novo* fatty acid synthesis in the mammary gland. *OLR1* mRNA was initially identified in bovine aortic endothelial cells (Sawamura *et al.* 1997), where it is highly expressed compared with other tissues. The association of $OLR1_{g.8232C>A}$ with long chain fatty acids might reflect the high expression of *OLR1* in heart tissue and the origin of these long chain fatty acids, i.e. the bloodstream. This SNP in the 3′ UTR of *OLR1*, or another SNP that is in linkage disequilibrium, might influence the expression level of *OLR1* and therefore affect long chain fatty acid composition of milk. This hypothesis is supported by the finding that the expression of *OLR1* transcripts was lower in AA cows than in CC cows (Khatib *et al.* 2006).

PPARGC1A

PPARGC1A plays a central role in the activation of nuclear hormone receptors and transcription factors regulating energy homeostasis. Weikard *et al.* (2005) identified *PPARGC1A* as a candidate gene for milk production traits and found an association between a SNP in this gene ($PPARGC1A_{c.1892+19C>T}$, located in intron 9) and milk-fat yield in German Holstein cattle. Allele frequency of $PPARGC1A_{c.1892+19C>T}$ is in line with the previous reports (Weikard *et al.* 2005; Khatib *et al.* 2007b). $PPARGC1A_{c.1790+514G>A}$, in intron 8, was newly identified in this study. We could not confirm the reported effects of $PPARGC1A_{c.1892+19C>T}$ on milk production traits. Weikard *et al.* (2005) found an association of the C allele with lower milk-fat yield in German Holsteins. However, this finding could also not be confirmed in an American Holstein population (Khatib *et al.* 2007b).

Both SNPs in *PPARGC1A* showed significant effects on milk-fat composition, however, not on the same fatty acids and unsaturation indices. The effect on the unsaturation indices might reflect the role of PPARG transcription factors in the complex regulation of fat synthesis and metabolism. The finding that PPAR agonists are able to increase *SCD1* mRNA levels in humans, mice and rats suggests that PPARs are able to regulate *SCD1* (Popeijus *et al.* 2008). As the SCD1 enzyme is involved in the desaturation of saturated fatty acids into *cis*9-unsaturated fatty acids, PPARs might have an effect on unsaturation indices by their regulation of *SCD1*.

PRL

PRL is a hormone secreted by the anterior pituitary with multiple functions, including critical roles in mammary gland development, lactogenesis and galactopoiesis. A synonymous SNP in exon 4 of *PRL* has been genotyped in various dairy cattle populations. The allele frequency of this $PRL_{g.8398G>A}$ SNP in the Dutch HF population is in line with the literature (allele frequencies of several studies are reviewed by Brym *et al.* 2005). We did not find an effect of this SNP on milk production traits. The previous reports on association analyses with milk-fat percentage or milk-fat yield did not show consistent results (Dybus 2002; Brym *et al.* 2005; Dybus *et al.* 2005).

$PRL_{g.8398G>A}$ shows an effect on C10:0 and C12:1. PRL has a critical role in mammary gland development, lactogenesis and galactopoiesis. The mechanism by which PRL exerts its effects on milk-fat composition is not known, but this could be via *STAT5A*, which is shown to be regulated by PRL (Welte *et al.* 1994).

STAT5A

STATs are transcription factors known to play an important role in cytokine signalling. The STAT5A protein was initially identified as a mammary gland factor and mediates the action of PRL on, among others, milk-protein gene expression. SNPs in the *STAT5A* gene have been associated with milk production traits, including milk-fat percentage in Jersey, Polish Black-and-White and US Holstein cattle (Brym *et al.* 2004; Flisikowski *et al.* 2004; Khatib *et al.* 2008). Allele frequencies of a SNP in intron 9, $STAT5A_{g.9501G>A}$, are in line with those reported by Brym *et al.* (2004). We were not able to confirm the association of the A allele of this SNP with a higher fat percentage, as reported by Brym *et al.* (2004) in Jerseys but not Polish Black-and-White cattle.

$STAT5A_{g.9501G>A}$ is associated with medium chain fatty acids and indices, where the A allele is associated with more saturated and less unsaturated medium chain fatty acids, and lower indices. STAT5 proteins are implicated in a wide variety of signalling events (Hennighausen & Robinson 2008). Studies with transgenic mice have shown that STAT5 proteins have a function in the regulation of mammary tissue development (Teglund *et al.* 1998), and several *in vitro* and *in vivo* studies have also indicated a role for STAT5A in adipogenesis and fat cell function (Floyd & Stephens 2003; Richter *et al.* 2003).

To summarize, only $FASN_{g.17924A>G}$ and $OLR1_{g.8232C>A}$ showed significant effects on milk-fat percentage in our cattle population (Table 2). The majority of reported associations between other SNPs and milk-fat percentage or milk-fat yield could not be confirmed in this study, nor could effects on other milk production traits such as protein percentage or milk yield. This could be explained by the generally smaller number of animals used in the previous studies. The fact that we are not able to confirm the results of previous studies could suggest that these SNPs are not the causal mutations. Alternatively, mutations do not always have the same allele substitution effects in all populations or breeds, depending on the specific genetic and environmental background.

All genotyped SNPs showed a significant effect on milk-fat composition at the 5% level (Table 2). We are aware of the fact that we performed multiple tests and that some of the effects might be significant simply by chance: based on the total number of performed tests, the expected number of false positives would be 9.1 when using a 5% significance threshold, and 1.6 considering a 1% significance threshold. However, we presented all effects with a *P*-value smaller than 5%. Furthermore, our true Type I errors will be slightly higher than the ones we reported, as we used pre-corrected data in our analyses.

Linkage disequilibrium

Two of the analysed SNPs are located in the *FASN* gene, and two SNPs in the *PPARGC1A* gene. Both *FASN* and *STAT5A* genes are located on BTA19, about 8 Mb apart. To indicate if the effects of the SNPs in *FASN* and *STAT5A*, and of the SNP in *PPARGC1A* were partly explaining the same variation, r^2 was calculated. The r^2 for the SNP in *FASN* was 0.14, and the r^2 for the SNP in *PPARGC1A* was 0.09. For the SNP in *FASN* and *STAT5A*, r^2 was below 0.01. These low r^2-values suggest that the effect of one SNP explained variation in the other SNPs only to a small extent.

QTL for C14:0 on BTA19

In a previous study, we performed a genome-wide scan to detect QTL for bovine milk-fat composition using linkage analysis (Schennink *et al.* 2009; Stoop *et al.* 2009). This genome-wide scan consisted of 849 cows representing five large and two small paternal half-sib families in a weighted across-family regression. The cows included in the genome-wide scan were part of the 1905 animals used in this study. Significant QTL were detected on BTA6, 14, 15, 16, 19 and 26. On BTA6, where *PPARGC1A* is located, a significant QTL was detected for C6:0 and C8:0. However, SNPs in *PPARGC1A* were not associated with C6:0 or C8:0. On BTA19, where *FASN* and *STAT5A* are located, a significant QTL was detected for C14:0. All three SNPs in *FASN* and *STAT5A* showed significant association with C14:0 in the current association study. Both *FASN* and *STAT5A* are located within the confidence interval for the QTL, at 58 and 62 cM respectively. To investigate whether the SNPs in *FASN* or *STAT5A* were contributing to the QTL for C14:0 on BTA19, the QTL analysis was repeated using phenotypes corrected for the SNP effect in *FASN* or *STAT5A*. Correction

of the phenotypes neither eliminated the QTL, nor decreased its significance substantially, suggesting that the SNP cannot (fully) explain the QTL detected. Subsequently, the genotypes of the seven bulls that sired the daughters in the genome-scan were analysed (Table 3). All seven sires were homozygous for $FASN_{g.16024G>A}$, implying that this SNP could not contribute to the QTL detected in the linkage study. Three out of the seven sires were heterozygous for $FASN_{g.17924A>G}$ (sire 5, 6 and 7) and $STAT5A_{g.9501G>A}$ (sire 4, 5 and 6), indicating that these SNPs could be contributing to the QTL. However, sire 2, which is significantly segregating for the QTL, is homozygous for both $FASN_{g.17924A>G}$ and $STAT5A_{g.9501G>A}$. This last observation shows that although $FASN_{g.17924A>G}$ and $STAT5A_{g.9501G>A}$ might contribute to the QTL for C14:0 on BTA19, the QTL cannot solely be explained by these two SNPs. An alternative explanation could be that the SNPs in *FASN* and *STAT5A* are in linkage disequilibrium with a causal mutation and that these SNPs are not causal SNPs.

In conclusion, we showed effect of SNPs in *FASN*, *OLR1*, *PPARGC1A*, *PRL* and *STAT5A* on milk-fat composition in Dutch HF cattle. However, we were not able to confirm the effect of these SNPs on milk production traits, except for *FASN* and *OLR1*. This finding stresses the need for replication of association studies in different cattle populations.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Means, standard deviations (SD) and heritabilities[1] (h^2) of traits under study, as measured on test-day morning milk samples from 1905 first-lactation Dutch Holstein-Friesian cows.

Table S2 PCR and extension primers[1] for direct sequencing and genotyping using SNaPshot assay.

Table S3 Description of the studied polymorphisms.

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