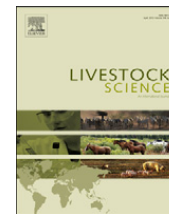




Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Association of genes involved in carcass and meat quality traits in 15 European bovine breeds



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ARTICLE INFO

Article history:

Received 5 August 2012

Received in revised form

31 January 2013

Accepted 27 February 2013

Keywords:

Bos taurus

Candidate genes

Meat quality

SNP

Association studies

ABSTRACT

Variations in meat quality traits are under complex genetic control and improvement has been hampered by the difficulty in their measurement. Several QTL have been reported for different meat quality related traits, but few genes have been described which explain large amounts of the phenotypic variation. The use of single nucleotide polymorphism (SNP) marker panels with predictive value for carcass traits have been evaluated for cattle and SNP are commercially available even though their predictive accuracy may be low in different breeds. To identify new molecular markers for meat quality, an association study was performed in 15 breeds of cattle using 389 SNP belonging to 206 candidate genes known to be involved in muscle development, metabolism and structure. Fifty-four SNP belonging to 20 different genes were found associated with different growth, carcass and meat quality traits. Some of them were novel associations and other were replications of known associations. Among the former, the gene-network associated with the calpain/calpastatin system was shown to be associated with meat texture, although small effects are found for the examined polymorphisms. Novel associations also included SNP in *AANAT* which was associated with collagen ($P=0.006$), *CAST* with fatty acid muscle composition ($P=0.00003$), *CYP1A1* with juiciness ($P=0.0005$), *DGAT2* with physical traits ($P=0.0009$) and lipid content ($P=0.01$) in muscle, *MADH3* with the myofibrillar fragmentation index (MFI) ($P=0.01$), *NEB* with weight ($P=0.00009$), *PCSK1* with juiciness ($P=0.002$), *PLOD3* with carcass performance ($P=0.0009$) and fatty acids ($P=0.04$), and *PGAM2* and *VIM* with post-mortem maturation ($P=0.00008$ and 0.000005 , respectively). These data provide a starting point to investigate the complex gene-networks underlying economically important traits which are of importance to the beef industry for the improvement of production efficiency and meat quality.

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1. Introduction

Meat quality traits are very complex, involve many genes and are greatly influenced by a variety of environmental factors. Being difficult and expensive to measure, they are not usually included in selection programs based on phenotypic performance. However, the identification of genetic markers for quality traits could provide the industry with

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the possibility to select for improved quality, while minimising the cost of trait recording. Many studies have identified QTL involved in meat quality related traits in beef cattle (e.g. Casas et al., 2000, 2003); however, the dissection of these QTL has not identified genetic variants explaining a large portion of phenotypic variance (Van Eenennaam et al., 2007). More recently, SNP within candidate genes have been tested for predictive value for carcass traits (e.g. Barendse, 2002; Buchanan et al., 2002; Page et al., 2002), and some commercial tests based on SNP marker panels are being proposed to breeders to genotype animals. However, to date few mutations within specific genes have been associated with the complex traits describing carcass and meat quality in cattle (see the review in Ibeagha-Awemu et al., 2008), and most of these SNP have relatively small effect, which may differ between breeds. It is unlikely that the genomic selection (GS) currently used to estimate breeding values for quantitative traits in dairy cattle (Luan et al., 2009), will be used in beef cattle populations due to small population sizes and lack of high accuracy estimated breeding values (EBV). Therefore, at least in the short term, genomic selection can be improved by extending the panel of SNP in the candidate loci and better estimating SNP effects in different populations.

In the study reported here, 389 SNP identified in 206 candidate genes possibly involved in muscle development, metabolism and structure (described in Williams et al., 2009) were tested for association with meat quality traits in 15 European cattle breeds.

2. Materials and methods

2.1. Animals

A total of 436 unrelated pure bred bulls and 539 parents (either sire, dam or both when available) belonging to 15 breeds were used. The breeds included specialised beef breeds, dairy breeds, and unimproved “local” breeds. The whole sample included 31 bulls and 30 parents Jersey, 27 bulls and 20 parents South Devon, 30 bulls and 26 parents Aberdeen Angus, and 29 bulls and 26 parents Highland from United Kingdom; 29 bulls and 39 parents Holstein, 29 bulls and 37 parents Danish Red Cattle, and 20 bulls and 20 parents Simmental, from Denmark; 30 bulls and 45 parents Asturiana de los Valles, 31 bulls and 42 parents Asturiana de la Montaña, 30 bulls and 44 parents Avileña-Negra Ibérica, and 31 bulls and 50 parents Pirenaica from Spain; 30 bulls and 45 parents Piedmontese, and 28 bulls and 13 parents Marchigiana from Italy; and 31 bulls and 47 parents Limousin, and 30 bulls and 55 parents Charolais from France.

Bulls from beef and local breeds were purchased between 6 and 9 months of age. Bulls of the dairy breeds were purchased as young calves (average 7 days for Jersey or 1–1.5 month for Holstein and Danish Red), and raised until 6 months when they were transferred to the standardised management protocol used for all the breeds. A uniform beef management system representative of those used in the European Union (EU) countries was used for all breeds to homogenise as far as possible influence of management and rearing system on meat quality (Albertí

et al., 2008). Efforts were made to standardise as much as possible rearing conditions (identical feeding, rearing in groups of individuals of the same breed, slaughter in same conditions or in the same day whenever possible and at same age, etc.).

Blood sampling and DNA extraction were performed in Williams et al. (2009).

2.2. Feed system

Bulls were fed a total mixed ration containing barley and soy bean with appropriate minerals and vitamins. All ingredients were mixed into a form that prevented selection using molasses up to 3–5% as a binding agent. Metabolisable energy of the ration was 12.5 kJ/kg and straw was available *ad libitum* to provide fibre. Bicarbonate was added to the ration to prevent acidosis. This diet was designed to achieve the slaughter weight of 75% of mature weight for each breed within a window of 13–17 months (Albertí et al., 2008).

2.3. Phenotypes measured

A comprehensive range of carcasses phenotypes were measured which fell into four categories: growth traits, measured on the live animal until slaughter, carcass measurements, both described in Albertí et al. (2008); physical variables; and sensory analysis previously described in Christensen et al. (2011). Phenotypes were used individually in the association tests or were integrated into trait groups e.g. ‘Taste Panel’ trait group, which included juiciness, flavour, and tenderness. All traits are listed in Table S1.

2.4. SNP in candidate genes

Selection of candidate genes and identification and genotyping of the SNP have been described in Williams et al. (2009). The association analysis was performed using 389 SNP with minor allele frequencies above 10% in the breeds investigated (Williams et al., 2009). These SNP were genotyped across the 436 bulls and their available parents.

2.5. Statistical analysis

Two association strategies were used: a population level analysis based on linear models accounting for the known population substructures, and a transmission-disequilibrium approach using parental information (TDT; Spielman et al., 1993). In the latter case the methodological and operational extensions included in the PBAT software (Lange et al., 2004) were applied.

The first association test was a linear model developed to take advantage of the known information on population structure. Population-based models can detect spurious associations due to population structure when this is unknown. In this study there is a clear partition of the whole sample, formed by the different breeds, and this information was taken into account to avoid false positives. Therefore, the linear model calculated Y_{ij} as the

phenotype of individual j in breed i , for a biallelic marker with alleles A and a assuming that the phenotype follows the true model $M0$ given by $Y_{ij} = m_i + a_i G_{ij} + \varepsilon_{ij}^{(0)}$, where m_i is the phenotypic mean for breed i , a_i the additive effect of marker in breed when i , and G_{ij} take different values according to the genotype of each individual, assigning 1 for genotype AA , 0 for Aa and -1 for aa . $\varepsilon_{ij}^{(0)}$ are independent and identically distributed normal residuals, independent of G_{ij} .

Alternatively, a dominance scenario was explored with a full dominant model, and the same genotypes were coded as 0, 1, and 2. Many of the phenotypes were transformed (indicated in Table S1) to give normal distributions assumed for the linear model.

For the TDT analysis many parents were not available and therefore genotype data were missing. In addition many markers showed low heterozygosity. Together these problems meant that the number of informative trios was too low to achieve a statistically power for detecting associations with PBAT. The 1-TDT (Sun et al., 1999) approach, and its extension for quantitative traits (Sun et al., 2000) that allows for missing parents, was therefore adopted. The statistic $z(c) = (s_1(c) + s_2(c)) / \sqrt{\sigma_1^2 + \sigma_2^2}$ (where $s_1(c)$ $s_2(c)$ and $\sigma_1^2 \cdot \sigma_2^2$ are the conditional means (s) and variance (σ^2) when one (subscript 1) or both parents (subscript 2) are known) combines transmission counts and phenotypic values with their variances for trios with either one or two parents genotyped, and follows a normal distribution when the number of heterozygous parents is sufficiently large.

Another main problem to solve was multiple testing, as the number of traits measured and of markers genotyped was huge, and there were thousands of tests to be performed. The problem arises when using a traditional Bonferroni correction for the individual α -level, $\alpha' = (\alpha/n)$ or $\alpha' = 1 - (1 - \alpha)^{1/n}$, where n is the total number of tests. This corrections lead to very small individual α values, and consequently to a loss of power, to keep the global α at the desired level. Besides the α correction, a multivariate linear analysis was also performed as an alternative to reduce the dimension of the problem, looking for associations between the marker and the whole phenotypic group following the method of composed principal components (CPC, Mangin et al., 1998). Two different procedures were followed to adjust the α level.

First, the effective number of markers (Nyholt, 2004) was calculated. This can be understood as the number of markers that would suffice to explain the correlation structure of the total set of markers. That number was used to apply an alternative α correction to Bonferroni, proposed by Benjamini and Hochberg (1995) (see also Benjamini and Yekutieli, 2005; Sabatti et al., 2003). They introduced the concept of false discovery rate (FDR), which controls the expected value of type I errors, unlike the significance level, which controls the probability of having one single type I error. Obviously, controlling α implies controlling FDR, but not reversely. FDR is a less restrictive condition, which leads to higher power.

The other strategy used to account for multiple testing was resampling. Given $m \leq n$, the idea is to calculate the

global type I error obtained if the m contrasts associated to the m highest values of the statistic were rejected. Values of the test statistic were used instead of p -values to avoid the necessary normality assumptions for p -value calculation under the linear model, which in many cases were not verified, as mentioned above. To do that, permutations were made on the genotypes of the individuals within breeds while keeping their phenotypes fixed, thus generating samples under the null hypothesis.

Haplotype association analysis was performed on those genes with two or more markers for which a strong association was found with particular traits when the 1-TDT multi-allelic extension was applied. As the pedigree information was insufficient to determine the phase of markers, the haplotypes and their frequencies were estimated using Famhap v1.6 (Becker and Knapp, 2004; Table S2).

See Annex I for a more detailed explanation on the statistical analysis performed.

2.6. Gene network

Gene pathways were built (Fig. S1) using the association results of this study along with previously published gene functions and associations (Barendse, 2002; Casas et al., 2006; Goll et al., 1992; Harris et al., 2011; Lauer-Fields et al., 2002; Levin et al., 2007; Orho-Melander et al., 2002; Page et al., 2002; Patel and Lane, 1999; Poloz and O'Day, 2009; Reardon et al., 2010; Shioda et al., 2006; Singh et al., 2008; White et al., 2005; Zhang et al., 2007).

3. Results

Fifty-four SNP belonging to 20 different genes were found associated with different growth, carcass and meat quality traits, either individually (Table 1) or included in a haplotype (Table 2). Mean and standard deviations (s.d.) for the traits associated with different SNP in the 15 breeds are given in Table S3. Table S4 shows the allele frequencies per breed of the 44 polymorphisms found to be individually associated to different traits and Table S2 the haplotype frequencies of those genes with two or more markers for which a strong association was found when the 1-TDT multi-allelic extension was applied. Allele effects were estimated for the associations detected with the linear univariate model, whereas neither TDT nor linear approaches using the CPC correction allow for the calculation of effects. For univariate analysis, the allele showed in Table 1 is positively correlated with the trait.

These different association analyses performed on 15 European breeds revealed a total of 77 significant associations influencing growth, carcass and meat quality traits among 389 tested SNP. Among the results obtained, the hypothetical calpain/calpastatin system gene-network was shown to be associated with meat texture (Fig. S1). Novel associations also included the wide effects of *DGAT2* on carcass and physical traits, as well as on lipid content in muscle, connecting its effects on growth with both the calpain/calpastatin system and triacylglycerol (TG) synthesis, the links between *PRKAG2* and m-calpain activity, *CALM2* and calpastatin activity, *NEB* and weight, *CYP11A1* and *PCSK1* with juiciness, because of their novelty and

Table 1
Significant associations between individual SNP and different live, carcass and meat traits.

Locus symbol	dbSNP ^a	SNP location ^b	Significant trait associations ^c	Method ^d	Mean	Stand. Dev.	p-value	Alpha ^e	FDR ^f	Allele ^g	Effect	Effect/s.d.	
AANAT Chr 19	ss77831970	55918180 Intron 1	pH thaw	TDT			0.00001	0.00006		C			
			Total collagen	L	3.464	0.685	0.006	0.01		C	-0.039	-0.057	
			16ald	L	23.277	6.880	0.002	0.09		C	0.110	0.016	
			18ald	L	16.005	5.060	0.001	0.006		C	0.116	0.023	
			20:5 n-3	L	4.262	2.300	0.00001	0.00002		C	-0.109	-0.047	
			All fatty acids	L _{CPC}			0.001				0.04		
ACBP Chr 2	ss77831981	71562127 Intron 1	Flavour group ^h	L _{CPC}			0.002			0.03			
			9 Months pelvis width	LD	43.233	4.983	0.00008	0.0004		C	-0.059	-0.012	
CALM2 Chr 11	ss77832015	29425548 Intron 1	Flavour group	L			0.03		0.2				
			Calpastatin	L	31.620	4.339	0.0005	0.0007		G	-1.634	-0.377	
CAPN1 Chr 29	ss77832254	44068812 Intron 8	MFI ⁱ	L _{CPC}			0.001		0.2				
	ss77832259	44069063 Exon 9-NS 316aa Gly → Ala	MFI	L	3.653	0.159	0.061	0.069		G	-0.025	-0.157	
	ss77832258	44069177 Intron 9	MFI	L _{CPC}			0.002		0.2				
	ss77832257	44069247 Intron 9	MFI	L	3.653	0.159	0.01	0.013		C	-0.021	-0.132	
	ss77832255	44069255 Exon 10-S	MFI	L _{CPC}			0.003		0.2				
	ss77831763	44085642 Exon 14 530aa Ile → Val	MFI	TDT			0.0008	0.001		G			
CAST Chr 7	ss77832280	98541284 Intron 11	MFI	TDT			0.0029	0.0032		C			
			P 16:0	L	63.616	14.731	0.00003	0.001		T	0.025	0.002	
			P 18:0	L	52.115	9.012	0.0002	0.005		T	0.018	0.002	
CYP1A1 Chr 21	ss77832034	34318948 Intron 1	All fatty acids	L _{CPC}			0.05		0.2				
			Juiciness	L	4.761	0.728	0.0005	0.03		T	0.434	0.596	
			Drip loss	L	2.711	1.749	0.008	0.05		T	0.277	0.158	
DGAT2 Chr 15	ss77832040	55972514 3'UTR	Juiciness group ^j	L _{CPC}			0.001		0.2				
	ss77832040	55972522 3'UTR	Test panel ^k	L _{CPC}			0.003		0.1				
	ss77832040	55972514 3'UTR	12 Months weight	TDT			0.002	0.007		C			
	ss77832038	55972426 3'UTR	Calpastatin	L	31.620	4.339	0.005	0.009		C	-1.110	-0.256	
	ss77832038	55972426 3'UTR	Calpastatin	L	31.620	4.339	0.006	0.01		G	1.085	0.250	
	ss77832039	55972520 3'UTR	Calpastatin	L	31.620	4.339	0.007	0.01		G	1.073	0.247	
IGFBP2 Chr2	ss77831770	105363779 Intron 3	12 Months weight	TDT			0.001	0.002		C			
			Final weight	TDT			0.0009	0.001		C			
			All fatty acids	L _{CPC}			0.02				0.07		
			Flavour group	L _{CPC}			0.01				0.08		
			Maximun diameter	LD	9.601	1.046	0.00008	0.0003		G	-0.036	-0.034	
			MFI	L _{CPC}			0.01			0.2			
MADH3 Chr 10	ss77832358	13975390 Intron 6	Physical group ^l	L _{CPC}			0.005		0.2				
			MC2R Chr 24	ss77832362	44008662 Exon 2-5								
ME3 Chr 29	ss77831909	9161402 Intron 12	Juiciness	TDT			0.004	0.009		G			
	ss77831895	9161721 Intron 13	Test panel	L _{CPC}			0.02		0.1				
	ss77831890	9157508 Intron 10	A740 48 h	L	27.977	4.815	0.0006	0.002		A	-1.116	-0.232	
	ss77831891	9157590 Intron 10	Test panel	L _{CPC}			0.01		0.1				
	ss77831891	9157590 Intron 10	Test panel	L _{CPC}			0.008		0.1				
	ss77831729	9157431 Intron 10	Test panel	L _{CPC}			0.02		0.1				
	ss77831894	9161706 Intron 13	Test panel	L _{CPC}			0.004		0.1				
	ss77831896	9161840 Intron 13	Test panel	L _{CPC}			0.01		0.1				
	ss77831899	9161852 Intron 13	Test panel	L _{CPC}			0.009		0.1				
	ss77831900	9161859 Intron 13	Test panel	L _{CPC}			0.02		0.1				
	ss77831905	9160939 Intron 12	Test panel	L _{CPC}			0.01		0.1				
	ss77831907	9161069 Intron 12	Test panel	L _{CPC}			0.007		0.1				
	ss77831908	9161242 Intron 12	Test panel	L _{CPC}			0.02		0.1				

Table 1 (Continued)

MMP1 Chr 15	ss77831914	6124321 Intron 1	m-Calpain	TDT			0.001	0.05	G		
NEB Chr 2	ss77832091	44746820 Intron 141	15 Months weight	L	542.661	88.328	0.00009	0.0002	A	16.714	0.189
PCSK1 Chr 7	ss77831755	98156290 Exon 1-S	Juiciness group	L _{CPC}			0.002			0.19	
			Cook loss 48 h	L	28.972	2.105	0.0002	0.005	T	0.587	0.279
PGAM2 Chr 22	ss77831773	373009 Exon 1-S	COX	TDT			0.001	0.007	C		
	ss77831774	372582 Exon 2-S	A540 48 h	L	6.709	1.499	0.0001	0.006	A	-0.049	-0.033
			A560 48 h	L	8.423	2.015	0.00005	0.00009	A	-0.055	-0.027
			A580 48 h	L	6.522	1.554	0.00006	0.0002	A	-0.053	-0.034
			b 10 d	LD	13.775	1.934	0.00008	0.0001	A	-0.244	-0.126
PLOD3 Chr 25	ss77832218	36061724 Intron 11	Carcass ^m	L _{CPC}			0.005			0.3	
	ss77831757	36061548 Exon 11-S	Carcass	L _{CPC}			0.0009			0.2	
			All fatty acids	L _{CPC}			0.04			0.2	
PPM2C Chr 14	ss77831806	72682212 Exon 2-S	μ-Calpain	TDT			0.002	0.02	A		
	ss77831758	72681180 Exon 2-S	MFI	L _{CPC}			0.007			0.2	
PRKAG2 Chr 4	ss77832378	114895056 Exon 7-S	m-Calpain	L	5.288	0.881	0.0008		0.001	C	-0.286
	ss77832379	114894952 Intron 7	m-Calpain	L	5.288	0.881	0.0006		0.0009	C	-0.288
UCP3 Chr 15	ss77832403	54219574 Intron 2	12 Months weight	TDT			0.000004	0.00002	C		
			12 Pelvis width	TDT			0.0000002	0.00001	C		
			15 Pelvis width	TDT			0.00008	0.0001	C		
			9 Pelvis width	TDT			0.000009	0.00003	C		
			Carcass compactness index	TDT			0.00004	0.00009	C		
			Carcass weight	TDT			0.000004	0.00001	C		
			Final weight	TDT			0.00001	0.00008	C		
VIM Chr 13	ss77831736	31951829 Intron 7	b 10 d	LD	13.775	1.934	0.000005	0.00001	A	-0.513	-0.265
			L 10 d	L	41.337	3.832	0.00001	0.00005	A	-1.032	-0.269
			Physical group	L _{CPC}			0.002			0.1	

^a dbSNP Accession number.

^b Chromosome SNP position in the *Bos Taurus* UMD3.1 genome version, and SNP position in the gene. S: synonymous SNP; NS: non-synonymous SNP.

^c See Table S1 for trait descriptions.

^d Statistical methods applied: linear (L); linear dominance, LD; linear using CPC correction, L_{CPC}; transmission-disequilibrium test, TDT.

^e Significance level (α), which controls the probability of having one single type I error.

^f False discovery rate, which controls the expected value of type I errors.

^g Allele associated with the trait. For TDT analysis allele positively correlated with the trait.

^h Flavour group includes all fatty acids, all fatty acid ratios and flavour.

ⁱ Myofibrillar fragmentation index.

^j Juiciness group includes the traits pH at 3 and 24 h post mortem, pH on thawed samples at 10 days post mortem, drip loss, thaw loss, cook loss at 48 h and 10 days post mortem and juiciness.

^k Test panel includes all sensory analysis: tenderness, juiciness, beef flavour intensity, abnormal flavour intensity, texture, and overall appraisal.

^l Physical group gathers the traits pH at 3 and 24 h post mortem, pH on thawed samples at 10 days post mortem, drip loss, thaw loss, cook loss at 48 h and 10 days post mortem, maximum load cook at 48 h and 10 days post mortem, maximum load raw at 10 days, maximum load at 48 h and 10 days post mortem, stress20 at 48 h and 10 days post mortem, stress80 at 48 h and 10 days post mortem, L48 h, L10 d, a48 h, a10 d, b48 h, b10 d, chrom48 h, chrom10 d, hue48 h, hue10 d.

^m Carcass group gathers the traits dressing percentage (%), kidney fat weight (kg), fatness score (1–15), conformation score (1–18), carcass length (cm), internal depth of breast (cm), limb length (cm), limb width (cm), blockiness index, limb index (cm/cm), maximum diameter (cm), minimum diameter (cm), fat percentage and rib area (cm²).

Table 2
Significant associations between haplotypes and different live, carcass and meat traits.

Locus symbol	dbSNP (Allele1/Allele2) ^a	Haplotype ID and alleles	Significant trait associations ^b	p-value	Haplotype frequency
CAPN1	ss77832254 C/T ; ss77832259 C/G ; ss77832258 T/C ; ss77832257 C/del ; ss77832255 A/C ; ss77831763 G/A ; ss77832264 C/T ; ss77831762 G/C; ss77832261 G/A	3-CGCCACCA	MFI	0.00000003	0.268
ME3	ss77831909 G/A ; ss77831895 G/A ; ss77831890 G/T ; ss77831891 C/G ; ss77831729 G/A ; ss77831894 G/A ; ss77831896 C/T ; ss77831899 T/C ; ss77831900 T/C ; ss77831905 A/G ; ss77831907 A/G ; ss77831908 A/G ; ss77831893 G/A; ss77831901 Ins/G; ss77831904 G/A; ss77831730 C/T; ss77831898 A/G; ss77831910 C/T	5-AATGAGCTTAGAGInsGTAC	Texture	0.0009	0.310

^a In bold, SNP also found to be associated with some trait individually.

^b See Table S1 for trait descriptions.

quite considerable effect on those traits; and the association of *AANAT* with collagen, *CAST* with fatty acids (FA), *MADH3* with MFI, *PLOD3* with carcass performance and FA, and *PGAM2* and *VIM* with post-mortem maturation processes, which despite having small effects, are described here for the first time.

4. Discussion

Improving complex traits such as those related to meat organoleptic or health properties face a number of problems: measuring these traits is difficult to standardise and expensive (Simm et al., 2009) and therefore cannot be carried out on a large number of individuals; these traits often have low heritabilities and beef cattle have a long generation period. These factors make genomic selection impractical and the beef industry is currently using a candidate gene approach to select animal on genotypes at relatively few loci that have been identified with an effect on meat traits. Such tests are now being offered commercially (e.g. Igenity or GeneSTAR). However, these few loci account for only a small amount of the variation in the traits considered, and their effects cannot be extended to all *Bos taurus* breeds (Allais et al., 2011). Testing a wider range of candidate loci, selected for their physiological role in traits related to meat quality in a wide range of breeds, will provide the industry with new information to improve selection strategies.

More than 200 phenotypes including growth rate, weight, carcass length, muscle and fat composition, collagen amount, marbling score, instrumental tenderness, fatty acid profiles, water loss, pH, taste qualities (texture, flavour, juiciness), etc. (Table S1), were measured in 436 individuals belonging to 15 European cattle breeds, and 389 SNP corresponding to 206 candidate genes (Williams et al., 2009) were tested for associations with these traits, either individually or as groups of related traits. The association analysis identified 77 significant associations. Although some of the genes associated with traits affecting meat quality show relatively large effects, namely *PRKAG2* and *CALM2* explained in each case 5% of the variation in m-calpain and calpastatin activity respectively, *CYP1A1* with 10% of the variation in juiciness,

DGAT2 with 3.5% of the variation in calpastatin activity, *PCSK1* with 2% of the variation in cook loss, and *NEB* with 3% of the variation of the trait weight at 15 months age, most of the associations identified had an overall low effect. The meat quality associated traits measured are all polygenic and for some traits a very large number of genes contribute to its control. The selection of the panel of genes for testing was based on the knowledge of the underlying physiology on the biology of muscle and fat cell and the biochemistry of the muscle; however this knowledge is incomplete, so important genes and pathways are likely to have been overlooked. In addition from the panel of candidate genes no polymorphisms were identified in some of the genes. Therefore many genes which may have contributed to variations in meat quality traits were not tested.

The low number of associations may be due to the stringency of the correction for the high number of traits and SNP analysed. Several statistical aspects had to be addressed to solve this problem. First and foremost, the association tests to be applied had to be chosen and matched to experimental design. Then the problem of genotyping errors and incompatibilities had to be solved, as well as multiple testing, which became an important issue given the large number of phenotypes and markers involved. The traditional Bonferroni α correction led to very small individual α values and consequently to a loss of power. For this reason, a multivariate linear analysis was also performed as an alternative to reduce the dimension of the problem, looking for associations between the marker and the whole phenotypic group following the method of CPC (Mangin et al., 1998). The different approaches used are not exclusive and allowed finding associations, although lower in number than expected. This was performed by using a linkage disequilibrium test through a TDT approach which was limited by the small number of usable trios, either because of lack of genotyped parents or due to lack of heterozygous individuals for some markers, which led to an insufficient power of detection using PBAT software. A trio approach allowing for missing parents was then adopted, the 1-TDT (Sun et al., 1999) and its extension for quantitative traits (Sun et al., 2000), increasing the performance of the analysis. To consider the clear partition of the whole sample formed by 15 very different breeds, a linear model

under the additive model was performed to avoid false positives and the test $H_0 \equiv \beta = 0$ vs. $H_1 \equiv \beta \neq 0$ has been probed (data not shown) to be valid as a test of association.

4.1. Genes affecting tenderness

Calpains enzymes are responsible for protein breakdown in meat post-mortem whereas calpastatin is an inhibitor of calpains, thus the balance between these enzymes determines the rate of muscle breakdown after slaughter and meat tenderness. Several polymorphisms in calpain (*CAPN1*, *CAPN3*) and calpastatin (*CAST*) were included in the analysis, however, none were associated with differences in the activity of calpain or calpastatin in muscle. SNP ss77832259 in the isoform calpain-1 (*CAPN1*) results in a glycine to alanine substitution at amino acid position 316 in the ninth exon of the gene and has been associated with meat tenderness in cattle (Page et al., 2002; White et al., 2005) with the G allele associated with tougher meat. In this study the G allele was found associated with a reduction of MFI by 0.157-s.d. which is coherent with a tougher meat as MFI is an indicator of resolution of rigour, with higher values representing a greater fibre breakdown and hence more tender meat. SNP ss77832259 is in strong linkage disequilibrium with SNP ss77832257 which was also found to be associated with MFI. SNP ss77831763 that caused an isoleucine to valine substitution at position 530 has also been previously associated with meat tenderness (Page et al., 2002), and in the present study was also associated with MFI, as well as 4 more SNP not previously described to do so. Also concerning haplotypes, 1 out of the 6 haplotypes described for *CAPN1* was found associated with MFI. Polymorphisms in the calpastatin (*CAST*) gene have been associated with post-mortem effects on meat tenderness (Barendse, 2002), juiciness (Casas et al., 2006), water-holding capacity and colour in cattle (Reardon et al., 2010), however in the present study variations in this gene were associated with the total amount of FA and the phospholipid saturated FA 16:0 and 18:0 in muscle. Calpains have been implicated in the regulation of adipocyte differentiation (Patel and Lane, 1999) and the isoform calpain-10 has been associated with high free FA levels (Orho-Melander et al., 2002). Thus *CAST*, which is a calpain inhibitor, may influence the amount of FA in muscle through the regulation of calpain activity.

Other genes are found to influence the activity of calpastatin in the muscle as are *CALM2* and *DGAT2*, whereas *MMP1*, *PPM2C* and *PRKAG2* were linked to m-calpain or μ -calpain muscle enzyme activity. However, the pathways through which all these genes connect their activity with the calpain/calpastatin system are not totally clear and a hypothetical network is shown in Fig. S1. Specifically calmodulin 2 (*CALM2*) is a Ca^{2+} -binding protein that regulates many different protein targets (Cohen and Klee, 1988). There is an evidence that calmodulin can activate certain calpain substrates (calcineurin A and fodrin) and also plays a role in their cleavage (Shioda et al., 2006). CaM-binding protein (CaMBP) is homologous to calpastatin and shows inhibitory activity against m-calpain (Singh et al. 2008). The interaction

between calmodulin, its target CaMBPs, and calpain is involved in meat tenderisation (Poloz and O'Day, 2009). In the present study *CALM2* was found to be associated with the amount of calpastatin in muscle with the SNP ss77832015 accounting for a 5% ((effect·mean of the trait⁻¹)·100). In the case of *DGAT2*, this gene encodes the enzyme acyl-CoA:diacylglycerol acyltransferase 2, which catalyses the final step of TG synthesis in eukaryotes. Together *DGAT2* and *DGAT1* account for most of the TG synthesis that influences fat deposition in animals (Harris et al., 2011). Over-expression of *DGAT2* in glycolytic muscle of mice increases the content of TG, ceramides, and unsaturated long-chain fatty acyl-CoAs (Levin et al., 2007). In this study, an association between *DGAT2* and the amount of FA in muscle was detected. The increase of FA directly influences flavour. In addition two SNP in the 3'UTR of *DGAT2* showed significant associations with live weight at 12 months and final weight. These results are in agreement with those of Zhang et al. (2007), who found associations between several polymorphisms in *DGAT2* and growth traits. Three SNP at 3'UTR of *DAGT2* were also associated with calpastatin activity in muscle, which may link *DGAT2* and growth traits through the influence of the synthesis of TG and of calpastatin activity, which inhibits calpains and ultimately influences muscle growth (Goll et al., 1992). However the favourable haplotype was not shown in any breed (Table S2).

The matrix metalloproteinase-1 (*MMP1*) gene plays a key role in collagenolysis and has been directly implicated in extracellular matrix breakdown (Lauer-Fields et al., 2002). In the data presented here, SNP ss77831914 was found to affect the activity of m-calpain in muscle. Protein phosphatase 2C (*PPM2C*) encodes a family of proteins with multiple isoforms and diverse cellular functions, including Ca^{2+} -dependent signal transduction, DNA repair, mitogen-activated protein kinase systems, and dephosphorylation of cofilin (reviewed in Hishiya et al., 1999). In the present study *PPM2C* was found to be associated with the levels of μ -calpain and MFI. Although little is known about the activity of proteins phosphatases, the association with μ -calpain is supported by the role of calpains on MFI, which is an indicator of post slaughter protein breakdown. Finally, the *PRKAG2* gene, which encodes the AMP-activated protein kinase (AMPK) regulatory gamma 2 subunit, was found to be associated with m-calpain levels in muscle, linking *PRKAG2* to metabolic pathways that determine post-mortem meat maturation. Alleles of the SNP tested accounted for 5% of the observed variation in m-calpain activity. However, the mechanism underlying this linkage is unclear. In chickens this gene is expressed at higher levels in the muscles of lean compared to fat chickens (Sibut et al., 2008). Lower *PRKAG2* expression has been found in individuals exhibiting extremely high muscle glycogen content which is related to post-mortem pH, a key factor controlling meat quality (Sibut et al., 2011).

MADH3 (also known as *SMAD3*) was associated with variations in MFI. *MADH3* down-regulates myostatin (Ge et al., 2011) which is an inhibitor of muscle growth. A modification in the activity of the myostatin gene (*GDF8*)

by *MADH3* may explain its influence in tenderness as myostatin also affects this trait through the reduction of collagen content and muscle fibre section (Allais et al., 2010).

4.2. Genes affecting physical and carcass traits

Arylalkylamine N-acetyltransferase (*AANAT*) was associated with differences in total FA content in muscle and the 'Flavour group' (a phenotypic group that includes all FA measurements, all FA ratios and flavour test). *AANAT* encodes the penultimate enzyme in the production of melatonin and has been linked so far to reduction of lipid peroxidation and hence protection of long chain PUFA in biological systems (Fagali and Catalá, 2007), and specifically omega-3 PUFA muscle content in cattle (Pérez et al., 2010). In the present study the C allele of the SNP ss77831970 increases n-hexadecanal (16ald) and n-octadecanal (18ald) fatty aldehydes which are saturated fatty aldehydes that have been described as key components of beef flavour (Resconi et al., 2010) due probably to their strawberry and coconut fragrances respectively, but decreases eicosapentaenoic acid (EPA 20:5 n-3), a beneficial omega-3 FA. The same allele was also found to affect collagen content and pH thaw; this last trait has been linked to microfibrillar breakdown (Abbasvali et al., 2012).

The melanocortin 2 receptor *MC2R* was associated with the 'Physical group' of traits, which includes pH and drip loss, and tenderness traits such as shear force. All of these traits are related to post-mortem muscle biochemical changes. *MC2R* is selectively activated by adrenocorticotrophic hormone (ACTH) and angiotensin II (Beuschlein et al., 2001), which induces synthesis and secretion of glucocorticoids. Previous studies have shown that an increase in plasma concentrations of corticosterone due to acute post-mortem stress suppresses protein synthesis capacity along with the augmentation of protein catabolism in skeletal muscles and thus decrease of both glycogen stores and buffer capacity, thus pH (Gao et al., 2008).

Two SNP in *PGAM2* (ss77831773 and ss77831774) were associated with the colour traits L^* and b^* . The inseparable relationship between beef colour and pH is widely accepted (Mancini and Hunt, 2005). The association between *PGAM2* and drip loss percentage has already been observed in pigs (Fontanesi et al., 2003). *PGAM2* affects the activity of cytochrome-c oxidase (COX) in muscle. COX catalyses the transfer of electrons from ferrocycytochrome c to oxygen in the mitochondria and therefore is involved in mitochondrial respiration (Rich et al., 2002). Cytochrome c also has a significant role in the activation of apoptosis (Liu et al., 1996) and alters generation and elimination of H_2O_2 (Pereverzev et al., 2003; Zhao et al., 2003). Therefore, the observed effects of *PGAM2* on colour traits may be explained by its interaction with COX activity.

The ss77831736 SNP of the myofibrillar protein vimentin (*VIM*), was associated here with b^* and L^* at 10 days with an effect of 0.27-s.d., and the 'Physical group' of traits, all of which are related to post-mortem maturation processes and meat quality. Beef with higher pigment content has lower L^* and higher redness (a^*) (MacDougall,

1982). Caspase mediated proteolysis of vimentin has been shown to cause the irreversible disruption of intermediate filaments and the generation of a pro-apoptotic amino-terminal cleavage product that signal cell death (Byun et al., 2001). Thus, changes in the expression of *VIM* may modify apoptosis and hence affect meat physical traits such as colour, pH, drip loss or tenderness (Laville et al., 2009).

The SNP ss77831981 of the acyl-CoA-binding (*ACBP*) gene was found associated with the 'Flavour group' and with the live trait pelvis width at 9 months. Recently *ACBP* has also been shown to affect body shape, muscle strength and growth rate in mice (Neess et al., 2011). *ACBP* encodes a 10-kDa intracellular protein which is highly conserved throughout evolution (Burton et al., 2005) and is known to protect acyl-CoA esters from hydrolysis, to block acyl-CoA inhibition of a number of enzymes, and to donate acyl-CoA esters to phospholipid, glycerolipid, and cholesteryl ester synthesis (Chao et al., 2003). Deficiency of *ACBP* results in increased levels of 18:0 acyl-CoA esters and a decrease in the amount of total 26:0 FA (Schjerling et al., 1996). Therefore *ACBP* is involved in FA elongation and the influence of this gene on the trait 'Flavour group' is clearly consistent with its physiological roles.

SNP ss77831770 in the insulin-like growth factor-binding protein 2 precursor (*IGFBP2*), which appears to be associated with a slight decrease of the maximum diameter, measured as the length of an imaginary line that divides the area of the *Longissimus dorsi* of the 6th rib in medium-lateral sense and correlated with the salable proportion of meat in the carcass. *IGFBP2* regulates the bioavailability of IGF1 and IGF2 (Jones and Clemmons, 1995), and circulating *IGFBP2* concentrations in cattle are negatively correlated with body weights and IGF1 concentrations (Pagan et al. 2003).

Variations in nebulin (*NEB*) gene were associated with weight at 15 months age. This trait had a mean of 542.7-kg but with a large s.d. (88.3). The SNP ss77832091 in *NEB* had an effect of 0.19 s.d., and the A allele was associated with and increase of 16.7-kg. *NEB* encodes a giant protein component of the cytoskeletal matrix (Stedman et al., 1988) and has been so far associated with meat toughness (Lee et al., 2008).

Two SNP of procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 precursor (*PLOD3*) were associated with the phenotypic group 'Carcass'. This association may be related to the activity of lysyl hydroxylase 3 (LH3) which is encoded by *PLOD3*. LH3 is correlated with collagen formation and hence with skeletal muscle development (Heikkinen et al., 2000). The SNP ss77831757 in *PLOD3* was also linked with the total amount of FA in muscle which may be related to the loss of muscular proteins which is negatively correlated with the adipose mass in muscle (Anderson and Kunkel, 1992).

The mitochondrial uncoupling protein 3 (*UCP3*) SNP ss77832403 was positively correlated with live weight, pelvis width and final weight, and carcass weight and carcass compactness index. The SNP tested in the current study is located in intron 2, however, a SNP in intron 3 of the *UCP3* gene has also been associated with average daily gain, efficiency of growth, and feed conversion ratio (Sherman et al., 2008).

4.3. Genes affecting juiciness

CYP1A1 was associated with juiciness and drip loss as individual traits but also when they are considered in the 'Juiciness group' or within the 'Test panel' trait groups. The effects of the T allele of SNP ss77832034 both on juiciness and drip loss were large, representing about 10% of the variation on these traits. *CYP1A1* belongs to cytochromes P450 enzyme family, which catalyse the oxidation of substrates including lipids and steroid hormones (Ioannides, 1996). The mechanism through which this gene may influence juiciness is not clear but this is one of the most important association found in this study and should be investigated further as an individual bearing allele T at SNP ss77832034 will show higher juiciness.

Twelve polymorphisms out of the 18 SNP analysed in the mitochondrial NADP⁺-dependent (m-NADP-ME) malic enzyme (*ME3*) show association: one with juiciness and the 'Test panel' group, another one with absorbance at wavelength 740 at 48 h, and 10 with the 'Test panel' group. In addition, 1 haplotype was linked with texture measurements. Malic enzyme catalyses the oxidative decarboxylation of malate to pyruvate using NADP⁺ as a cofactor (Loeber et al., 1994). The cytosolic NADP⁺-dependent (c-NADP-ME) (*ME1*) isoform has been associated with back-fat thickness and muscle pH in pigs (Vidal et al., 2005), and with cooking loss, pH at 24 h post-mortem and eye muscle area in cattle (Zhou et al., 2011).

Also, prohormone convertase 1 (*PCSK1*) was associated with the 'Juiciness group' of traits and cooking loss at 48 h. *PCSK1* processing pathway is involved in converting POMC into the peptide hormones ACTH, α -melanocyte stimulating hormone (α -MSH) and β -endorphin (Hook et al., 2009). The association of *PCSK1* with juiciness may be mediated through ACTH and β -endorphin, increasing glucocorticoids through the activation of *MC2R*, gene which has shown here to be associated with meat quality, and influencing glucose metabolism (Liu et al., 2006), which also affects post-mortem maturation processes, respectively.

The currently incomplete knowledge of both muscle biology and the biochemical events that occur after slaughter make it impossible to explain some of the observed associations which should be further validated. For example, *MMP1*, *PPM2C* and *PRKAG2* were found here to influence calpain activity in muscle, although no link between these genes and calpain has been published so far. On the other hand, the physiological role of *AANAT* in lipid peroxidation and hence the effect of this gene on FA seems logical; however, why the gene should also affect collagen content and pH on thawed samples is difficult to explain. Some associations that may have been expected, based on previous published reports and the biochemical roles of the genes products, were not validated here. For example, many works report effects of *CAST* on tenderness or related traits (e.g. Barendse, 2002; Casas et al., 2006; Reardon et al., 2010), whereas other studies do not observe this expected association: on one hand, Jiang et al. (2009) examined shear force of cooked steak and taste panel tenderness rating in a Wagyu \times Limousin population and did not see any effect of *CAST*; on the other hand, Allais et al. (2011) observed associations

between polymorphisms of *CAST* and meat tenderness but these associations appeared to be breed-specific. In any case, the high stringency of the correction applied to the high number of traits and SNP analysed together with the 15 different breeds and the relatively few individuals within each population, does possibly miss some positive results and lowers the success of this candidate gene approach, but allows a view on the issues that should be addressed when starting this kind of association studies.

In conclusion, despite most of the 77 significant associations reported here had an overall low effect, some of the genes show considerable and novel effects as are *PRKAG2* on the variation of m-calpain activity, *CALM2* on calpastatin activity, *CYP1A1* on juiciness, *DGAT2* on calpastatin activity, *PCSK1* on cook loss, and *NEB* on the trait weight at 15 months age. Novel associations also included SNPs in *AANAT*, *CAST*, *CAPN1*, *CYP1A1*, *MADH3*, *MMP1*, *PLOD3*, *PGAM2*, *PPM2C*, and *VIM*, with different traits related to meat tenderness, juiciness, physical characteristics and carcass performance. Therefore, the data presented here offer scientific community a starting point from which to study some complex gene-networks underlying economically important traits, and when validated, provide molecular tools to the beef industry to improve production efficiency and meat quality.

Conflict of interest statement

None of the authors of the manuscript entitled "Association of genes involved in carcass and meat quality traits in fifteen European bovine breeds" have potential conflicts of interest which should be disclosed.

Acknowledgements

This work was supported by an EC grant QLK5—CT2000-0147.

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Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.livsci.2013.02.020>.

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