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Genome-wide comparative analyses for selection signatures indicate candidate genes for between-breed variability in copper accretion in sheep

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ABSTRACT

The problem of copper (**Cu**) intoxication and deficiency continues to impact economic gains and animal welfare in sheep husbandry. This study investigated the ovine genome for regions and potential genes under selection for Cu accretion between sheep breeds. For this, we compared ovine single nucleotide polymorphism (**SNP**) data of three Cu-susceptible breeds with three Cu-tolerant breeds. After merging SNP data of breeds and removal of related individuals, a total of 229 sheep and 45 640 autosomal SNPs were left. Then, we selected 14 individuals per breed into two datasets (datasets 1 and 2) for analysis of selection signatures using the Fixation index, cross-population extended haplotype homozygosity and haplotype-based FLK methods. Selection regions shared by both datasets detected by at least two methods revealed regions on OAR 4, 8 and 11 containing 54 candidate genes under selection for Cu accretion. Enrichment analysis revealed that 19 gene ontologies and 1 enriched Kyoto encyclopaedia of genes and genomes pathway terms were associated with the candidate genes under selection. Genes such as *TP53*, *TNFSF13*, *TNFSF12*, *ALOX15*, *ALOX12*, *EIF5A* and *PREP* are associated with the regulation of Cu homeostasis, programmed cell death or inflammatory response. We also found an enrichment of arachidonate 15-lipoxygenase activity, arachidonate 12-lipoxygenase activity and ferroptosis that influence cellular inflammation and cell death. These results shed light on ovine genomic regions under selection for Cu accretion and provide information on candidate genes for further studies on breed differences in ovine Cu accretion.

Implications

Copper intoxication and deficiency in sheep may be fatal and result in severe economic losses to sheep farmers. Sheep breeds vary widely in their accumulation of copper with the attendant need for varying levels of copper supplementation to prevent intoxication and deficiency. In this study, we investigated the genetic basis for differences in copper accretion between copper-tolerant and copper-susceptible sheep breeds by assessing signatures of selection. We observed candidate regions and genes that may help to understand the genetic basis for variability in copper accretion and reduce the incidence of copper-related diseases in sheep husbandry.

Introduction

Copper (**Cu**) is a trace mineral element that is essential for the function of several enzymes (Suttle, 2010). However, it results in toxicity and sudden death when consumed in excess. Among ruminants, sheep are known to be impaired in their ability to excrete excess Cu from the liver, thereby leading to increased susceptibility to Cu intoxication (Haywood et al., 2005). Cu poisoning in sheep ensues from exposure to toxic or non-toxic levels of Cu through food ingestion that lead to Cu accretion in the liver, as reviewed by Borobia et al. (2022). On the other hand, some sheep breeds are reportedly prone to Cu deficiency when compared with others (Wiener, 1979). Several studies show that body Cu levels depend not only on dietary Cu (van der Berg et al., 1983) but also on the age (Woolliams et al., 1982), on the breed (van der Berg et al., 1983), and on the presence of Cu antagonists such as molybdenum (Mo) (Suttle and Field, 1983). Existing documented information on

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breed differences in liver Cu accumulation dates back to the 1960 's. An initial study by Wiener and Field (1969) revealed significantly lower fitted liver Cu values for Scottish Blackface (14.3) compared to Welsh Mountain sheep (49.7) fed on the same pasture. Variations in liver (antilog of least squares means of $\log_{10}$ liver Cu concentration) and blood (μg/100 ml) Cu concentrations reported by Wiener et al. (1976) were low for Scottish Blackface (Liver Cu = 24.7, blood Cu = 92.3), intermediate for Cheviot (Liver Cu = 31.5, blood Cu = 99.2) and high for Welsh Mountain (Liver Cu = 65.8, blood Cu = 111.3), under similar environmental conditions, age and dietary Cu content. Likewise, Woolliams et al. (1982) found liver copper levels (mg Cu /kg DM) highest in Texel (1491.5), followed by Suffolk (1116.1), intermediate levels for East Friesian (754.2) and Finnish Landrace (766.7) and again low levels for Scottish Blackface (567.1) sheep fed similar Cu concentration in the diet. Similar findings by other authors confirm differences between breeds in hepatic Cu accretion for sheep when exposed to the same dietary Cu level (van der Berg et al., 1983; Suttle et al., 2002). All these studies suggest that Cu accretion is influenced by genetic factors.

Haywood et al. (2005) identified that hepatocytes of the Cu-susceptible North Ronaldsay sheep have a higher sensitivity to oxidative injury than the Cu-tolerant Cambridge sheep. Additionally, the report revealed that antioxidant protein-1 was increased 4–5 fold in the hepatic cytosol of the North Ronaldsay (but not Cambridge) sheep implying increased sensitivity of the mitochondria to Cu-induced oxidative stress. Simpson et al. (2006) also pointed to the involvement of mitochondria in the sensitivity of this sheep breed to Cu-induced oxidative stress. This report identified a high expression of Cu-responsive proteins such as cathepsin D, ferritin light chain, peroxiredoxin 3 and plasma retinol-binding protein in North Ronaldsay sheep whereas high levels of epoxide hydrolase and methionine adenosine transferase were observed in Cambridge sheep. These findings indicate that some biological processes modulated by genes influence Cu susceptibility or tolerance among sheep breeds.

Furthermore, several studies identified the involvement of genes such as copper transporter 1 (*CTR1*), chaperone for superoxide dismutase 1 (*CCS*), antioxidant 1 (*ATOX1*), synthesis of cytochrome c oxidase 1 (*SCO1*), synthesis of cytochrome c oxidase 2 (*SCO2*), metallothionein (*MT*), copper-transporting ATPase 1 (*ATP7A*) and copper-transporting ATPase 2 (*ATP7B*) in cellular Cu transport, circulation and distribution (Tao and Gitlin, 2003; Leary et al., 2004; Lutsenko et al., 2007; Barry et al., 2010; Lutsenko, 2010; Kaler, 2013). A comparison of the Cu-tolerant Simmental with the less Cu-tolerant Angus cows showed only for *CTR1 a* significant difference in liver expression, but not for other Cu-metabolism genes such as *ATOX1, ATP7A* and *ATP7B* (Fry et al., 2013).

The genome-wide single nucleotide polymorphism (**SNP**) genotyping allowed the development of various methods in order to identify selection signals within the genome. Selection signatures are genomic regions that were the main targets of selective breeding and may correspond to the differences in breed/populations (Saravanan et al., 2020). For this study, we employed genomic scans of the Fixation index ($\mathbf{F_{ST}}$) (Flori et al., 2009), cross-population extended haplotype homozygosity (**XP-EHH**) (Sabeti et al., 2007) and haplotype-based FLK (**hapFLK**) (Fariello et al., 2013) methods that allow the localisation of candidate selection sweeps within the genome. Unlike other methods such as Tajima's D and composite likelihood ratio which detect intra-population selection signals, the methods selected for this study aid the detection of inter-population selection signatures (Saravanan et al., 2020). Both XP-EHH and hapFLK statistics are based on homozygosity and differentiation of haplotypes, respectively. In combination with the genomic F_{ST} scan, this allows the detection of selection signals that overlap across methods.

Despite available information on the Cu accretion status of some sheep breeds, there has been no genome-wide approach trying to unravel the genetic architecture influencing between-breed variability in the Cu accretion of sheep. We recently published a study that identified candidate genes associated with liver Cu concentration within the Merino Land sheep (Adeniyi et al., 2023). However, genes and genetic regions associated with breed-dependent variations in Cu accretion of sheep have not yet been investigated. In this study, genome-wide information from the SNP genotypes and three different approaches (F_{ST}, XP-EHH and hapFLK) were used in comparative analyses to identify selection signals associated with Cu accretion in sheep. For this purpose, we grouped sheep breeds according to susceptibility or tolerance to Cu using available published information on breed differences in liver Cu accumulation. The aim of this study was to reveal genes and genomic regions that influence between-breed variation in Cu accretion and aid in the selection for reduced incidence of Cu toxicity or deficiency in sheep.

Material and methods

Animal and genomic data collection

Illumina Ovine 50 K SNP BeadChip genotyping data was retrieved from published sources for the sheep breeds including Scottish Texel (n = 80), Finnsheep (n = 96), Irish Suffolk (n = 55), Scottish Blackface (n = 56) (Kijas et al., 2012) and Welsh Mountain Hill flock (n = 24) (Beynon et al., 2015). Additionally, new SNP data for Scottish Blackface (n = 43) and German Grey Hearth (n = 14) are included in the dataset (Supplementary Table S1). These datasets were merged using PLINK v1.90 (Chang et al., 2015) and checked for relatedness which was estimated using –related option in KING v2.2.4 (Manichaikul et al., 2010). Prior to this, chromosomes and positions of SNPs were referred to the Oar_v4.0 *Ovis aries* genome assembly using the commands --update-map and --update-chr in PLINK v1.90. We excluded one of each pair of sheep within a breed with an Identity-by-descent > 0.30. A total of 229 sheep and 45 640 autosomal SNPs were left for further analysis (Supplementary Table S1).

Breed grouping for selection signature analysis

Prior to the performance of F_{ST}, XP-EHH and hapFLK analysis, the sheep breeds in our data were grouped according to Cu accretion, namely Cu-susceptible and Cu-tolerant based on the published reports (Wiener and Field, 1969; Herbert et al., 1978; Woolliams et al., 1982; van der Berg et al., 1983; Meyer and Coenen, 1994; Borobia et al., 2022). The breeds Scottish Texel and Irish Suffolk were considered as Cu-susceptible, whereas Finnsheep and Scottish Blackface were regarded as Cu-tolerant breeds (Woolliams et al., 1982; van der Berg et al., 1983) (Table 1). In addition, we classified the Welsh Mountain Hill flock and German Grey Hearth as Cu-susceptible and Cu-tolerant, respectively. This is based on published information comparing the Welsh Mountain Hill flock with Scottish Blackface (Wiener and Field, 1969; Wiener et al., 1976; Herbert et al., 1978), and German Grey Hearth with Texel (Meyer and Coenen, 1994) (Table 1). To avoid samples size bias due to the lower n for the German Grey Hearth (n = 14), Welsh Mountain Hill flock (n = 14) and Scottish Texel (n = 20) breeds, after merging and removal of related samples, a subset of our data consisting of all Welsh Mountain Hill flock and German Grey Hearth samples, as well as 14 selected sheep from each of

Table 1
Overview of published information on differences in liver copper (Cu) accretion (mg Cu/Kg DM) for Cu-tolerant and Cu-susceptible sheep breeds in this study.

Sources	Cu content of diet (mg Cu/kg DM)	Breeds[1]						Days of feeding
		Cu-tolerant			Cu-susceptible			
		Finnsheep	Blackface	German Hearth	Texel	Suffolk	Welsh Mountain	
Woolliams et al. (1982)	12	408.8	291.6	–	695.6	615.2	–	91
Woolliams et al. (1982)	20	766.7	567.9	–	1 491.5	1 116.1	–	91
van der Berg et al. (1983)	10	225	–	–	775	–	–	103
van der Berg et al. (1983)	34	486	–	–	1 652	–	–	103
Herbert et al. (1978)	30	–	240	–	–	–	316	105
Wiener and Field (1969)[2]	–	–	14.3	–	–	–	49.7	–
Wiener et al. (1976)[3]	–	–	24.7	–	–	–	65.8	168
Meyer and Coenen (1994)[4]	–	–	–	< 50	> 500	–	–	–

[1] This column contains liver Cu values for all breeds measured in mg Cu/Kg DM except stated otherwise.
[2] This row contains fitted liver copper values. Fed the same pasture.
[3] This row contains the antilog of least square means of log10 liver Cu concentration. Fed the same pasture.
[4] Over 60% of German Hearth sample were < 50 mg Cu/Kg DM whereas over 47% of Texel sheep were > 500 mg Cu/Kg DM. Fed the same pasture.

the other four breeds (Finnsheep, Irish Suffolk, Scottish Blackface and Scottish Texel) were included for analysis of selection signatures (dataset 1). We repeated the analyses with a different subset of Finnsheep, Irish Suffolk, and Scottish Blackface samples (dataset 2). For Scottish Texel samples in dataset 2, we included 8 samples from the first data subset to the 6 Scottish Texel samples remaining after initial selection for dataset 1. Both datasets contain 84 individuals.

Quality control of datasets

The data subsets (Supplementary Table S2) were filtered for quality control by the removal of loci with minor allele frequency (< 1%), SNPs with a low call rate (< 95%) and animals with more than 5% of missing genotypes using PLINK v1.90. A total of 45 080 and 45 076 autosomal SNPs remained in the datasets for genomic-based determination of selection signals after quality control for datasets 1 and 2, respectively (Supplementary Table S2). In addition, no sample was excluded from both datasets after quality control.

Population structure analysis

After quality control and linkage disequilibrium pruning using PLINK v1.90 (--indep-pairwise), datasets 1 and 2 including 42 107 and 42 150 autosomal SNPs (Supplementary Table S2), respectively, were analysed by principal component analysis (**PCA**) with the *dudi.pca* function of the package "ade4" (Dray and Dufour, 2007) in R Statistical Software v4.2.3 (R Core Team, 2023) and plotted by using the R v4.2.3 "GGPLOT2" package (Wickham, 2016). Pairwise distances between samples were calculated with the *dist.gene* function of the "ape" R v4.2.3 package (Paradis et al., 2004) and visualised in a neighbour-joining tree constructed with the Splitstree4 software v4.15.1 (Huson and Bryant, 2006).

Selection signature analysis

F_{ST} analysis comparing the Cu-susceptible (Scottish Texel, Irish Suffolk and Welsh Mountain Hill flock) group with the Cu-tolerant (Finnsheep, Scottish Blackface and German Grey Hearth) group was performed on quality control data subsets (datasets 1 and 2, Supplementary Table S2) using the --fst function in PLINK v1.90. The resulting F_{ST} values were Z transformed to normalise its distribution using the formula: $ZFST = (x - \mu) / \sigma$, where x refers to the F_{ST} value for each SNP, μ is the mean of all F_{ST} values and σ is the SD of all F_{ST} values as described by Eydivandi et al. (2021), and averaged for SNPs in non-overlapping windows of 500 Kb. The averaged ZFST values were plotted across the chromosome with the exclusion of regions containing less than five SNPs. The top 1% of informative windows were defined as candidate selection signals.

Likewise, a comparison between Cu-susceptible (Scottish Texel, Irish Suffolk and Welsh Mountain Hill flock) and Cu-tolerant (Finnsheep, Scottish Blackface and German Grey Hearth) breed groups for standardised XP-EHH scores were computed with the ies2xpehh function in R package REHH (Gautier and Vitalis, 2012) using haplotype information and default settings. Prior to this, integrated extended haplotype homozygosity (iES) values were determined using scan_hh function in the R "REHH" package with the option 'polarised = FALSE'. Haplotypes were estimated with fastphase 1.4 (Scheet and Stephens, 2006) by applying the following options for each chromosome: −T10 −C25 −Ku40 −Kl10 −Ki5 −Km1000 −H100, where T is the number of the start of EM algorithm, C is the number of iterations of EM algorithm, Ku is the upper limit of number of clusters, Ki is the upper and lower limit of number of clusters, Ki is the interval between values of number of clusters, Km is the number of SNP loci used for cross-validation and H is the number of haplotype samples, respectively. For determination of candidate selection signals, standardised XP-EHH scores were transformed into $pXP\text{-}EHH = -\log[1-2|\Phi XP\text{-}EHH - 0.5|]$ in which Φ (XP-EHH) represents the Gaussian cumulative distribution function, which was determined by the R function "pnorm". We defined potential selection signals as regions with a minimum of four SNPs within the top 1% of the pXP-EHH distribution equivalent to |XP-EHH| score of ± 2.79 and ± 2.77 for datasets 1 and 2, respectively. XP-EHH scores across all autosomes were plotted with the GGPLOT2 package (Wickham, 2016) in R v4.2.3.

Using the quality control data subsets, we performed a genome-wide scan for selection signatures with the hapFLK (Fariello et al., 2013). For this analysis, we calculated Reynolds distance between the groups (Cu-susceptible vs Cu-tolerant) using the *gene.dist* function in the "hierfstat" R package (Goudet, 2005) and converted the result to kinship matrix with available R script from https://forge-dga.jouy.inra.fr/documents/222. Analysis of hapFLK statistics was performed with the hapflk v1.2 software (Fariello et al., 2013) with the inclusion of the kinship matrix and other parameters such as −K 15 (number of clusters as earlier determined from haplotype estimation with fastphase software), --nfit = 1 (number of Expectation-Maximisation (EM) runs) and --ncpu = 2 (number of central processing units). Furthermore, we performed the scaling as well as *P*-value calculation of the hapFLK statistics with the available Python script (scaling_chi2_hapflk.py) obtained from https://forge-dga.jouy.inra.fr/documents/588. The top 1% of the

distribution of the hapFLK statistic was defined as candidate regions of selection signatures. Of these, only regions with a minimum of four significant SNPs were considered as regions under selection.

Gene identification and enrichment analysis

For all methods used in this study, selection signals found in both datasets were scanned for genes in the ovine genome assembly (NCBI: *Ovis aries*, ARS-UI_Ramb_v2.0). Subsequently, we selected genes that overlapped across a minimum of 2 methods for analysis of potential biological significance. We examined the functional enrichment of the selected genes using the Database for Annotation, Visualization and Integrated Discovery (**DAVID**) software (https://david.ncifcrf.gov, accessed on 07 March 2024) (Huang et al., 2009b, 2009a; Sherman et al., 2022). Additionally, we investigated Gene Ontology (**GO**) and Kyoto encyclopaedia of genes and genomes (**KEGG**) pathway terms. GO terms including biological process, cellular component and molecular function were accessed. Enriched GO and KEGG pathway terms were considered significant at *P*-value $\leq$ 0.05 and FDR $\leq$ 0.05 (Benjamini and Hochberg, 1995) before and after correction for multiple testing, respectively. Additionally, a Venn diagram showing a number of jointly and separately identified genes across datasets and methods was constructed with the "ggvenn" package in R v4.2.3 (R Core Team, 2023).

Results

Population structure and phylogenetic tree

In order to investigate the reliability of the grouping of sheep breeds into Cu-susceptible (Scottish Texel, Irish Suffolk and Welsh Mountain Hill flock) and Cu-tolerant (Finnsheep, Scottish Blackface and German Grey Hearth), we analysed the population structure and phylogenetic relationship of the breeds using PCA and neighbour-joining tree. The top two principal components (PC1 and PC2) of the PCA explain 5.95% and 5.08% (Fig. 1a) of the genetic variance for dataset 1, respectively. Similar percentages were obtained for dataset 2 (PC1, 5.45%; PC2, 5.04%) (Fig. 1b). In both PCA plots, PC1 distinguished Irish Suffolk from other breeds whereas Scottish Texel is separated from other breeds by PC2. However, the PCA plots (Fig. 1a and b) showed that Cu-tolerant breeds are clustered together, even though the Welsh Mountain Hill flock breed was near this cluster. The neighbour-joining tree for both datasets revealed that all breeds are distinct from each other and are clustered on the neighbour-joining plot in accordance with the Cu status grouping of breeds analysed in this study (Fig. 2a and b).

Genomic regions and genes under selection

After averaging Z-transformed F_{ST} values and removal of windows with less than five SNPs, we kept a total of 4 662 and 4 668 informative windows with an average of 10.02 and 10.01 SNPs per window (SD = 2.13 for both datasets) for datasets 1 and 2, respectively. To eliminate spurious regions of selection in our dataset, we compared selection signals between both datasets obtained by each method (Supplementary Table S3a, S3b, S4a, S4b, S5a and S5b) and selected common regions of selection for further analysis. Following this, overlapping signals of selection were observed in 18, 8 and 3 regions identified by F_{ST}, XP-EHH and hapFLK, respectively (Tables 2, 3 and 4). These regions under selection were found on OAR 1, 2,3,4,5,7,8,11,12,13 14,16 and 20 (Tables 2, 3 and 4). Two regions on OAR 4 and 8 were observed to be jointly identified as selection regions by F_{ST} and XP-EHH methods, and a region on OAR 11 was significant with all methods (Tables 2, 3 and 4; Fig. 3a and b). A total of 139, 96 and 113 genes are located within overlapping selection regions identified by F_{ST}, XP-EHH and hapFLK methods, respectively (Fig. 4a, b and c). Of these, 54 genes are in regions of selection identified by a minimum

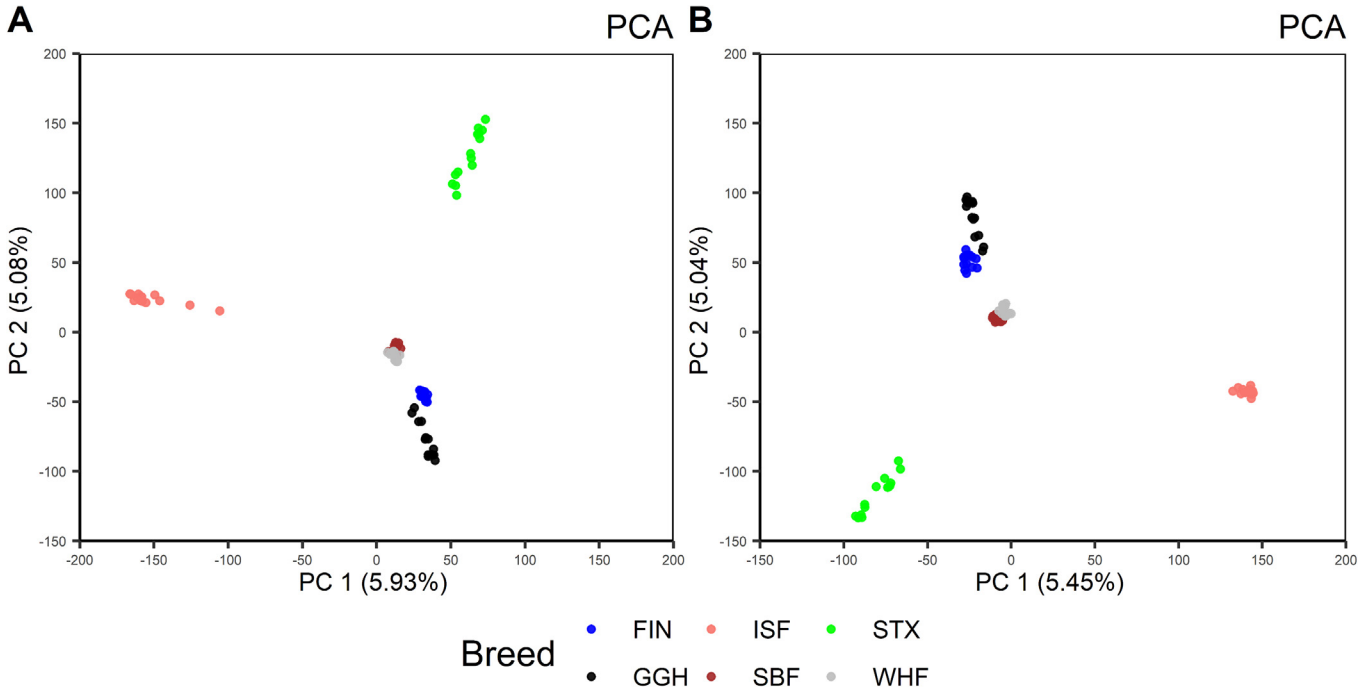

Fig. 1. Principal component analysis (PCA) plots of the six sheep breeds: Three copper-susceptible (STX = Scottish Texel; ISF = Irish Suffolk; WHF = Welsh Mountain Hill flock) and three copper-tolerant (FIN = Finnsheep; SBF = Scottish Blackface; GGH = German Grey Hearth) using (a) dataset 1 and (b) dataset 2.

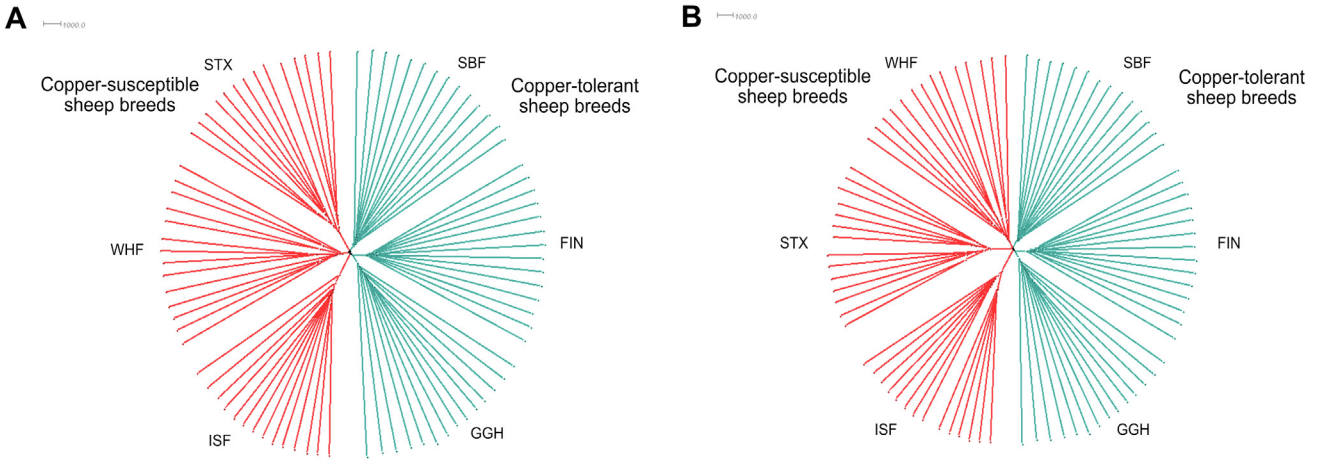

Fig. 2. Neighbour-joining tree plot of the six sheep breeds: Three copper-susceptible (STX = Scottish Texel; ISF = Irish Suffolk; WHF = Welsh Mountain Hill flock) and Three copper-tolerant (FIN = Finnsheep; SBF = Scottish Blackface; GGH = German Grey Hearth) using (a) dataset 1 and (b) dataset 2.

Table 2
Overlapping regions and genes under selection for copper accretion in sheep detected by F_{ST} for datasets 1 and 2.

Chr	Start[1]	End	Length of region (Kb)	Genes[2]
1	110 858 992	111 331 819	472.83	*VSIG8, CFAP45, TAGLN2, IGSF9, SLAMF9, PIGM, LOC121820658, KCNJ9, KCNJ10, IGSF8, ATP1A2, LOC101118398, CASQ1, PEA15, DCAF8*
1	135 585 935	136 060 808	474.87	*NCAM2*
2	113 644 524	114 107 784	463.26	*HERC2, NIPA1, NIPA2, CYFIP1, TUBGCP5*
3	146 440 774	146 890 350	449.58	*CNTN1, MUC19, LRRK2, LOC121819176, LOC121819177, LOC121819178, LOC121819179*
3	217 657 380	217 963 277	305.90	*MRTFA, MCHR1, SLC25A17, ST13, XPNPEP3, DNAJB7*
4	70 190 366	70 392 090	201.72	*EVX1*
5	58 342 060	58 821 145	479.09	*ABLIM3, LOC105606707, AFAP1L1, GRPEL2, PCYOX1L, IL17B, MIR143, PIGY, CSNK1A1, ARHGEF37, LOC114114896*
7	58 760 956	59 176 863	415.91	*DTWD1, FAM227B, FGF7, GALK2*
7	59 416 935	59 686 761	269.83	*SHC4, EID1, CEP152*
7	100 844 593	101 219 708	375.12	*LOC101115969, LOC114115667, CTDSPL2, EIF3J, SPG11, PATL2, B2M, LOC114108604, LOC121820091, LOC101120839, TERB2, LOC114115674*
8	31 743 299	31 935 827	192.53	*LOC101110768*
9	46 826 171	47 170 495	344.32	*NCOA2*
10	29 071 493	29 545 928	474.44	*FRY, LOC101110773, RXFP2*
11	26 831 470	27 199 959	368.49	*DVL2, PHF23, GABARAP, LOC101118285, CTDNEP1, ELP5, CLDN7, SLC2A4, YBX2, EIF5A, GPS2, LOC105607811, NEURL4, ACAP1, KCTD11, TNK1, PLSCR3, TMEM256, NLGN2, SPEM1, SPEM2, SPEM3, TMEM102, FGF11, CHRNB1, ZBTB4, TMEM95, SLC35G6, POLR2A, LOC101122801, TNFSF13, SENP3, EIF4A1, CD68, MPDU1, SOX15, FXR2, SAT2, SHBG, ATP1B2, TP53*
12	48 869 405	49 280 407	411.00	*MEGF6, ARHGEF16, PRDM16*
13	52 522 167	52 799 399	277.23	*TGM3, STK35*
14	42 817 493	43 231 297	413.80	*CEP89, FAAP24, RHPN2, LOC106991603, GPATCH1, WDR88, LRP3, SLC7A10, CEBPA*
20	17 006 839	17 487 984	481.15	*DLK2, TJAP1, LOC121817410, LRRC73, YIPF3, POLR1C, XPO5, POLH, GTPBP2, LOC101115833, RSPH9, MRPS18A, LOC105603710, VEGFA*

Abbreviations: Chr = Chromosome; F_{ST} = Fixation index; XP-EHH = cross-population extended haplotype homozygosity; hapFLK = haplotype-based FLK.
[1] SNP positions are based on the *Ovis aries* ARS-UI_Ramb_v2.0 genome assembly in NCBI, SNP = single nucleotide polymorphism.
[2] The underlined genes were identified by a minimum of two methods (F_{ST}, XP-EHH and hapFLK).

of two methods with 40 genes located in regions identified by all methods (Fig. 4d).

Enrichment analysis of genes under selection

To assess the functional significance of candidate selection genes associated with Cu accretion, all 54 genes (Supplementary Table S6) identified by at least two methods were included in a functional enrichment analysis using the DAVID software, which recognised 49 from these genes (Supplementary Table S6). The results show that 19 GO and 1 KEGG pathway terms are significantly enriched (P-value $\leq$ 0.05) (Table 5). Four of the enriched GO terms were significant after P-value adjustment (FDR $\leq$ 0.05) (Table 5). The most significant GO term is associated with lipoxygenase activity (GO:0016165, linoleate 13S-lipoxygenase activity) whereas the significantly enriched KEGG pathway is related to ferroptosis (oas04216, ferroptosis). Other GO terms observed in this study are involved in transcription regulation and repression including GO:0045944 (positive regulation of transcription from RNA polymerase II promoter) and GO:0001227 (transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding). Additionally, the 4 GO terms significantly enriched after P-value adjustment (FDR $\leq$ 0.05) include lipoxygenase activity and arachidonic acid metabolism.

Discussion

Variability in Cu accretion between sheep breeds has been discussed and documented extensively, but the underlying genetic

Table 3
Overlapping regions and genes under selection for copper accretion in sheep detected by XP-EHH for datasets 1 and 2.

Chr	Start[1]	End	Length of region (Kb)	Genes[2]
1	111 331 819	111 499 739	167.92	*COPA, NCSTN, NHLH1, VANGL2, SLAMF6*
1	254 851 954	254 931 259	79.31	*EPHB1*
4	50 954 084	51 056 257	102.17	*NRCAM*
4	70 190 366	70 888 570	698.20	*EVX1, HOXA13, LOC105606592, HOXA11, HOXA10, LOC121819410, HOXA9, HOXA7, HOXA3, HOXA5, HOXA6, HOXA4, HOXA2, HOXA1, SKAP2*
8	31 097 538	31 935 827	838.29	*PRDM1, LOC101107359, LOC114116192, LOC101110768*
11	26 588 278	27 199 959	611.68	*ALOX15, LOC114116880, LOC101113251, ALOX12, LOC105607815, RNASEK, C11H17orf49, BCL6B, LOC101114882, SLC16A11, LOC101117600, ASGR2, LOC101116157, DLG4, ACADVL, DVL2, PHF23, LOC101118285, CTDNEP1, ELP5, CLDN7, SLC2A4, YBX2, EIF5A, GPS2, NEURL4, LOC105607811, ACAP1, KCTD11, TMEM95, TNK1, PLSCR3, TMEM256, NLGN2, SPEM1, SPEM2, SPEM3, TMEM102, FGF11, CHRNB1, ZBTB4, SLC35G6, POLR2A, LOC101122801, TNFSF13, SENP3, EIF4A1, CD68, MPDU1, SOX15, FXR2, SAT2, SHBG, ATP1B2, TP53*
13	62 866 739	63 435 585	568.85	*CDK5RAP1, SNTA1, LOC101109899, CBFA2T2, NECAB3, ACTL10, E2F1, PXMP4, ZNF341, LOC105606908, CHMP4B, RALY*
16	33 688 218	33 786 111	97.89	*MROH2B, C7*
16	52 902 038	53 068 320	166.28	*No gene*

Abbreviations: Chr = Chromosome; F_{ST} = Fixation index, XP-EHH = cross-population extended haplotype homozygosity; hapFLK = haplotype-based FLK,
[1] SNP positions are based on the *Ovis aries* ARS-UI_Ramb_v2.0 genome assembly in NCBI, SNP = single nucleotide polymorphism.
[2] The underlined genes were identified by a minimum of two methods (F_{ST}, XP-EHH and hapFLK).

Table 4
Overlapping regions and genes under selection for copper accretion in sheep detected by hapFLK statistics for datasets 1 and 2.

Chr	Start[1]	End	Length of region (Kb)	Genes[2]
11	25 809 503	26 772 177	962.67	*NLRP1, LOC105616457, MIS12, DERL2, DHX33, LOC101106021, RPAIN, NUP88, RABEP1, ZFP3, LOC101114793, KIF1C, INCA1, CAMTA2, SPAG7, ENO3, PFN1, RNF167, SLC25A11, GP1BA, CHRNE, LOC105607817, C11H17orf107, MINK1, PLD2, PSMB6, LOC121820578, GLTPD2, VMO1, TM4SF5, ZMYND15, CXCL16, MED11, PELP1, ARRB2, ALOX15, LOC114116880, LOC101113251, ALOX12, LOC105607815, RNASEK, C11H17orf49, BCL6B, LOC101114882, SLC16A11, LOC101117600, ASGR2*
11	26 831 470	27 542 320	710.85	*DVL2, PHF23, LOC101118285, CTDNEP1, ELP5, CLDN7, SLC2A4, YBX2, EIF5A, GPS2, NEURL4, LOC105607811, ACAP1, KCTD11, TMEM95, TNK1, PLSCR3, TMEM256, NLGN2, SPEM1, SPEM2, SPEM3, TMEM102, FGF11, CHRNB1, ZBTB4, SLC35G6, POLR2A, LOC101122801, TNFSF13, SENP3, EIF4A1, CD68, MPDU1, SOX15, FXR2, SAT2, SHBG, ATP1B2, TP53, WRAP53, EFNB3, DNAH2, KDM6B, TMEM88, NAA38, LOC101105010, CHD3, RNF227, KCNAB3, TRAPPC1, CNTROB, GUCY2D, LOC101121185, ALOX12B*
11	27 858 086	28 430 355	572.27	*NDEL1, MYH10, CCDC42, MFSD6L, RPL26, PIK3R6, PIK3R5, NTN1*

Abbreviations: Chr = Chromosome; F_{ST} = Fixation index; XP-EHH = cross-population extended haplotype homozygosity; hapFLK = haplotype-based FLK.
[1] SNP positions are based on the *Ovis aries* ARS-UI_Ramb_v2.0 genome assembly in NCBI, SNP = single nucleotide polymorphism.
[2] The underlined genes were identified by a minimum of two methods (F_{ST}, XP-EHH and hapFLK).

factors are not known. In this study, we performed signatures of selection analyses on two separate datasets (Datasets 1 and 2) using three methods (F_{ST}, XP-EHH and hapFLK) to identify candidate regions and genes under selection for Cu accretion in sheep. In the available literature, the Welsh Mountain Hill flock and German Grey Hearth were not compared directly with Texel and Scottish Blackface, respectively, but the reported separate comparisons of Welsh Mountain Hill flock and German Grey Hearth with a known Cu-tolerant (Scottish Blackface) and Cu-susceptible (Texel) breed, respectively, support our grouping. Moreover, the clustering of these breeds in the neighbour-joining tree (Fig. 2a and b) confirms the grouping of sheep breeds applied in this study. However, different regions or genes were recently reported by Adeniyi et al. (2023), who studied within-breed differences in hepatic Cu concentration whereas here we investigate between-breed variations in Cu accumulation. This suggests that differences in Cu accretion within the previously studied breed (Merinoland sheep) are controlled by other genes and pathways than in this study.

Within overlapping regions observed between both datasets used in this study, a candidate region (~1 Mb) on OAR11 (26.2–27.2 Mb) was identified as a potential region of selection influencing variation in Cu accretion among sheep breeds by a minimum of two methods. Genes located within this region include solute carrier family 2 member 4 (*SLC2A4*), tumour protein p53 (*TP53*), TNF superfamily member 13 (*TNFSF13*), TNF superfamily member 12 (*LOC101122801* or *TNFSF12*), arachidonate 15-lipoxygenase (*ALOX15*), arachidonate 12-lipoxygenase 12S type (*ALOX12*) and eukaryotic translation initiation factor 5A (*EIF5A*), which are involved in oxidative stress and apoptosis processes. Other candidate regions under selection harbour even-skipped homeobox 1 (*EVX1*) and prolyl endopeptidase (*LOC101110768* or *PREP*) located on OAR4 and OAR8, respectively.

Earlier reports have implicated TP53 in the transcriptional regulation of the synthesis of cytochrome c oxidase 2 (*SCO2*) gene that produces a protein required for Cu transportation to the cytochrome c oxidase (COX) complex (Matoba et al., 2006; Madan et al., 2011; Won et al., 2012). Matoba et al. (2006) showed that the regulation of mitochondrial respiration by TP53 is associated with its impact on SCO2 synthesis. This is not surprising due to the influence of SCO2 on the COX complex, an important site of cellular oxygen consumption during aerobic respiration (Timón-Gómez et al., 2018). In addition, various studies have reported the influence of TP53 on various forms of programmed cell death such as apoptosis (Zuckerman et al., 2009; Aubrey et al., 2018; Wang et al., 2022b) and ferroptosis (Li et al., 2020; Xu et al., 2023).

Ferroptosis is a regulated cell death that is dependent on reactive oxygen species accumulation induced by Fe^{2+}-oxidised lipids (Li et al., 2020). Though Fe is the main ferroptosis-inducing agent, Gao et al. (2021) reported that elesclomol induced ferroptosis in cells by degradation of ATP7A, which in turn increases the quantity

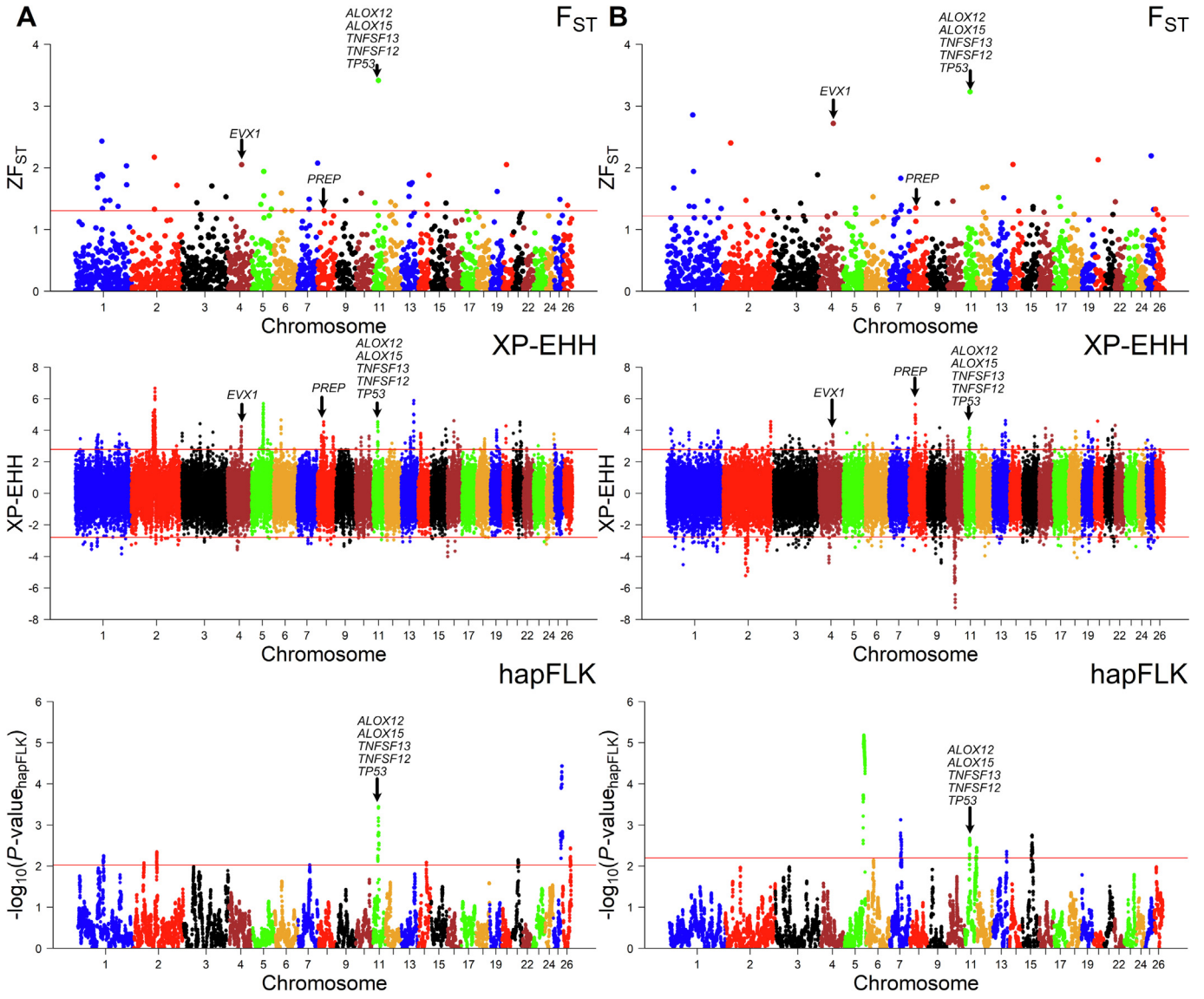

Fig. 3. Manhattan plots for Fixation index (F_{ST}), cross-population extended haplotype homozygosity (XP-EHH) and haplotype-based FLK (hapFLK) analyses of copper-susceptible vs copper-tolerant sheep breeds using (a) dataset 1 and (b) dataset 2. The red lines represent the top 1% of the distribution of each method.

of cellular Cu. The authors observed that increasing Cu accretion led to ROS accumulation and ferroptosis. Genes identified in this study including *TP53*, *ALOX12* and *ALOX15* have been associated with ferroptosis (Xu et al., 2023). This suggests that the sensitivity of cells to ferroptosis is associated with breed differences in Cu accumulation.

Recently, a novel type of cell death termed "cuproptosis" has been associated with Cu homeostasis and mitochondrial respiration (Chen et al., 2022; Li et al., 2022; Tang et al., 2022). Currently, TP53 is considered a potential regulator of pathways influencing cuproptosis (Xiong et al., 2023). According to Xiong et al. (2023), TP53 may facilitate cuproptosis by enhancing mitochondrial metabolism, regulating glutathione (GSH) levels and inhibiting glycolysis in cells. The process resulting in glycolysis inhibition may involve the downregulation of the *GLUT* genes (Kawauchi et al., 2008; Vousden and Ryan, 2009). Interestingly, the gene *SLC2A4* observed within the identified selection signal on OAR 11 is a known *GLUT* gene. Our findings indicate that *TP53* which regulates cuproptosis and SCO2 protein production may have a major effect on variation in Cu homeostasis and accumulation between sheep breeds.

On the other hand, *ALOX15* and *ALOX12* genes encode lipoxygenases that catalyse fatty acid peroxidation in cells resulting in inflammation (Chu et al., 2019; Snodgrass and Brüne, 2019; Benatzy et al., 2022). ALOX15 has been identified in the production of pro-resolving mediators that promote inflammation resolution (Wuest et al., 2012; Snodgrass and Brüne, 2019). These findings suggest that genes within the selection region may influence sensitivity to Cu-induced oxidative stress and cell death as observed by Simpson et al. (2006) in liver cells of Cu-susceptible North Ronaldsay sheep. Furthermore, the *TNFSF12* and *TNFSF13* genes identified in this study are associated with induction of apoptosis, regulation of angiogenesis and plasma cell survival (Locksley et al., 2001; Gaur and Aggarwal, 2003). Previous studies have reported that *TNFSF* genes encode inflammatory cytokines that regulate survival, proliferation, apoptosis and immune response of cells (Rath and Aggarwal, 1999; Idriss and Naismith, 2000).

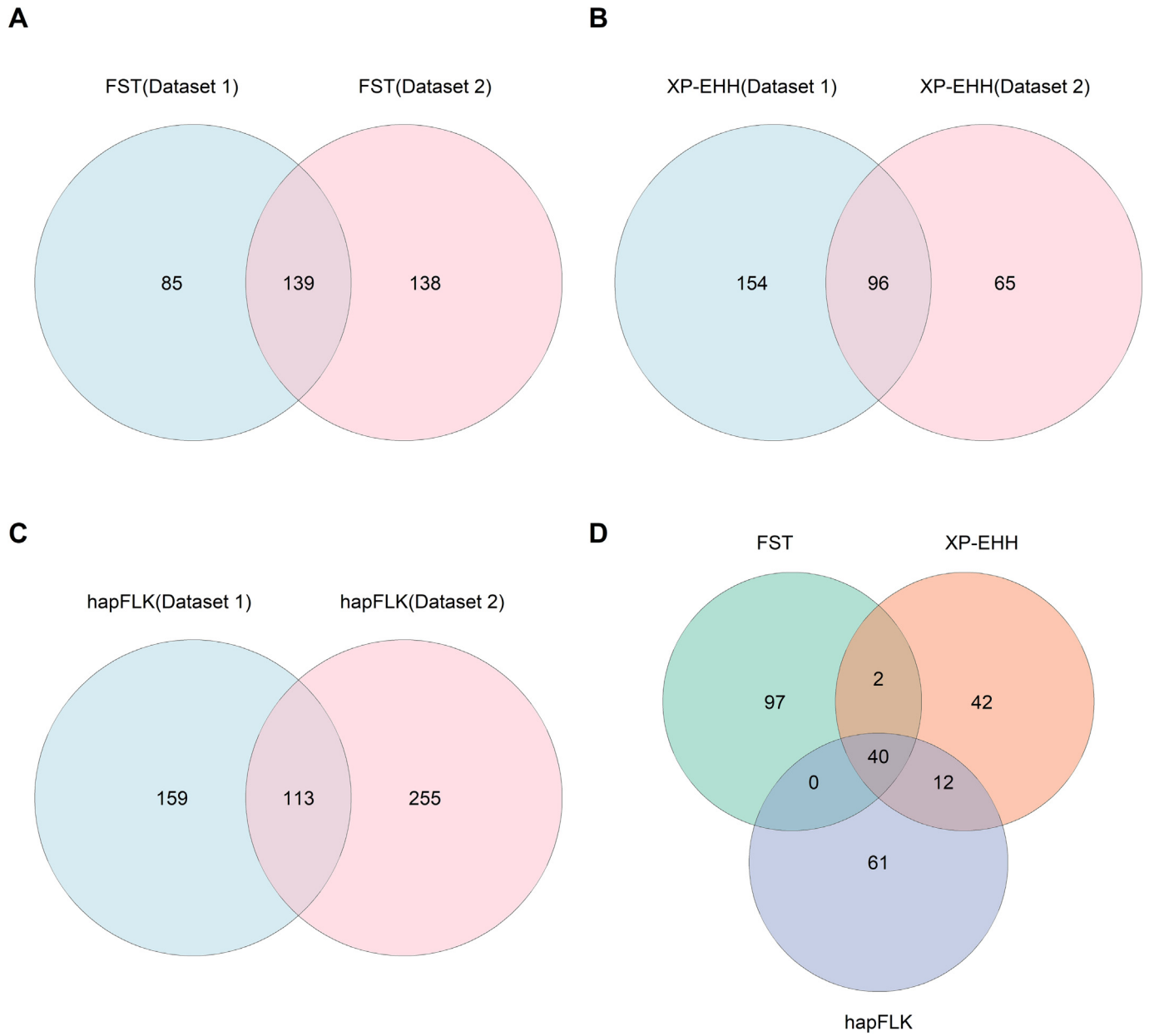

Fig. 4. Venn diagrams show the number of overlapping genes under selection for copper accretion in sheep between dataset 1 and dataset 2 for (a) Fixation index (F_{ST}), (b) cross-population extended haplotype homozygosity (XP-EHH), (c) haplotype-based FLK (hapFLK) methods, and (d) across F_{ST}, XP-EHH and hapFLK methods.

The TNFSF12 also known as TNF-like weak inducer of apoptosis (TWEAK) is recognised by a receptor termed fibroblast growth factor (FGF)-inducible molecule 14 (FN14, TNFRSF12A). TWEAK is a cytokine that modulates various cellular processes including angiogenesis, apoptosis, proliferation, survival, differentiation and inflammation after binding to FN14 (Winkles, 2008). TWEAK functions by binding to FN14, which is linked to several intracellular signalling pathways including TNFR-associated factor (TRAF), which initiates downstream signalling pathway such as nuclear factor-κB (NF-κB) (Wang et al., 2022a). The TWEAK/FN14 signal has been suggested to affect cellular response to tissue repair following injury (Vince et al., 2008; Dostert et al., 2019). Moreover, Tirnitz-Parker et al. (2010) reported that TWEAK in conjunction with FN14 facilitates hepatic regeneration during chronic injury via the NF-κB pathway. In another report, increased TWEAK and FN14 expression was associated with hepatic stellate cell (HSC) activation and higher levels of liver fibrosis in response to liver injury (Wilhelm et al., 2016). In addition, Wilhelm et al. (2016) found higher FN14 expression in activated HSCs than in quiescent cells. The impact of TWEAK/FN14 interaction has been described as a double-edged sword that facilitates the repair of liver progenitor cells in acute liver damage while contributing to the pathogenesis of chronic liver damage via induction of liver fibrosis (Wang et al., 2022a). Interestingly, hepatic Cu accumulation has been associated with progression in liver fibrosis (Hatano et al., 2000). A comparison of the North Ronaldsay (Cu-susceptible) and Cambridge (Cu-tolerant) sheep revealed that HSCs were activated in liver cells from North Ronaldsay and quiescent in Cambridge after excess Cu intake. Variation in hepatocyte TWEAK/FIN4 expression may be accountable for differences in HSC response to Cu-induced injury, which leads to varying stages of liver fibrosis development, as observed by Haywood et al. (2005). In addition, EIF5A has been identified as a pro-apoptotic protein in TNF-α mediated apoptosis (Taylor et al., 2004) and may regulate the activities of TP53 (Li et al., 2004; Taylor et al., 2007).

Table 5
Enriched GO and KEGG pathway terms determined by DAVID from genes identified in selection regions associated with copper accretion in sheep by a minimum of two methods (F_{ST}, XP-EHH and hapFLK).

Category	Term	Genes	*P*-value	FDR
GOTERM_MF	GO:0016165~linoleate 13S-lipoxygenase activity	*ALOX15, ALOX12, LOC101113251*	0.00	0.00
GOTERM_MF	GO:0004052~arachidonate 12-lipoxygenase activity	*ALOX15, ALOX12, LOC101113251*	0.00	0.00
GOTERM_BP	GO:0019372~lipoxygenase pathway	*ALOX15, ALOX12, LOC101113251*	0.00	0.02
GOTERM_BP	GO:0019369~arachidonic acid metabolic process	*ALOX15, ALOX12, LOC101113251*	0.00	0.04
GOTERM_CC	GO:0071339~MLL1 complex	*EIF4A1, SENP3, C11H17ORF49*	0.00	0.14
GOTERM_MF	GO:0043621~protein self-association	*PLSCR3, DVL2, TP53*	0.00	0.09
KEGG_PATHWAY	oas04216:Ferroptosis	*ALOX15, SAT2, TP53*	0.00	0.43
GOTERM_CC	GO:0016328~lateral plasma membrane	*CLDN7, DVL2, ATP1B2*	0.00	0.17
GOTERM_MF	GO:0050473~arachidonate 15-lipoxygenase activity	*ALOX15, ALOX12*	0.01	0.12
GOTERM_BP	GO:2001303~lipoxin A4 biosynthetic process	*ALOX15, ALOX12*	0.01	0.59
GOTERM_MF	GO:0003743~translation initiation factor activity	*EIF5A, EIF4A1, SENP3*	0.01	0.12
GOTERM_CC	GO:0005737~cytoplasm	*EIF4A1, SOX15, TNFSF13, ALOX12, KCTD11, ATP1B2, SPEM1, ELP5, TP53, SENP3, LOC101113251*	0.01	0.34
GOTERM_BP	GO:0043651~linoleic acid metabolic process	*ALOX15, ALOX12*	0.01	0.70
GOTERM_BP	GO:0007406~negative regulation of neuroblast proliferation	*KCTD11, TP53*	0.01	0.70
GOTERM_MF	GO:0001046~core promoter sequence-specific DNA binding	*POLR2A, TP53*	0.02	0.30
GOTERM_BP	GO:0016926~protein desumoylation	*EIF4A1, SENP3*	0.02	0.85
GOTERM_BP	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	*SOX15, DVL2, GPS2, EVX1, TP53*	0.02	0.85
GOTERM_CC	GO:0005829~cytosol	*FXR2, PLSCR3, ALOX15, TNK1, DVL2, GPS2, ALOX12, SLC2A4, ELP5, ZBTB4, TP53*	0.04	0.77
GOTERM_MF	GO:0005506~iron ion binding	*ALOX15, ALOX12, LOC101113251*	0.04	0.50
GOTERM_MF	GO:0001227~transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding	*BCL6B, ZBTB4, TP53*	0.05	0.50

Abbreviations: DAVID = Database for Annotation, Visualisation and Integrated Discovery software; F_{ST} = Fixation index; XP-EHH = cross-population extended haplotype homozygosity; hapFLK = haplotype-based FLK; GO = gene ontology; GOTERM = gene Ontology term; GOTERM_MF = molecular function; GOTERM_BP = biological process; GOTERM_CC = cellular component; KEGG_PATHWAY = Kyoto Encyclopedia of Genes and Genomes pathway; FDR = False discovery rate.

The *PREP* gene identified in this study has been associated with hepatic inflammation, oxidative stress and regulation of cathepsin B and D (Jiang et al., 2020; Zhang et al., 2021; Lin et al., 2023). Zhang et al. (2021) suggested that TP53 mediates the influence of PREP on the regulation of oxidative stress status in the liver. Furthermore, a transcriptional regulation of cathepsin B and D expression by PREP has been suggested (Lin et al., 2023). Interestingly, Simpson et al. (2006) reported an increase in cathepsin D for Cu-challenged North Ronaldsay (Cu-susceptible) sheep when compared with Cambridge sheep (Cu-tolerant). Therefore, we speculate that *TP53*, *TNFSF13*, *TNFSF12*, *ALOX15*, *ALOX12*, *EIF5A* and *PREP* genes have been under selection for Cu accretion in sheep.

Our study reveals that GO and KEGG pathway terms such as arachidonate 15-lipoxygenase activity, arachidonate 12-lipoxygenase activity and ferroptosis were enriched in this study. Genes contributing to these GO and KEGG pathway terms including *TP53*, *ALOX15* and *ALOX12* may influence Cu homeostasis (Madan et al., 2011; Won et al., 2012), programmed cell death (Chen et al., 2022; Xu et al., 2023) and response to inflammation (Snodgrass and Brüne, 2019). Our findings suggest that variability in response to hepatocellular inflammation and apoptosis may be responsible for breed difference in liver Cu accretion in sheep. This is in concordance with the finding that North Ronaldsay sheep (Cu-susceptible) have a higher mitochondrial sensitivity to Cu-induced oxidative stress than Cambridge sheep (Haywood et al., 2005; Simpson et al., 2006). The identified candidate genes and genomic regions responsible for differences in Cu accumulation between sheep breeds may allow the selection within susceptible breeds of individuals with reduced Cu accretion as well as the selection within breeds that are highly prone to deficiency of individuals with an improved Cu retention. This may be supported by an optimisation of the feeding. A limitation of this study was the relatively low number of sheep breeds with sufficient information on Cu accretion as well as available genomic data. To this end, further studies to investigate the Cu accretion status of other sheep breeds with available genomic data are needed to provide additional information on genetic factors influencing breed differences in Cu accumulation.

Conclusions

We compared sheep breeds based on the accumulation of Cu using selection signatures analysis methods including F_{ST}, XP-EHH and hapFLK. For this purpose, we used available ovine genomic SNP data to identify regions and genes associated with variations in Cu accretion between sheep breeds. The findings of this study suggest that OAR 4, 8 and 11 contain potential gene regions involved in Cu accretion. In these regions, we identified *TP53*, *TNFSF13*, *TNFSF12*, *ALOX15*, *ALOX12 EIF5A* and *PREP* as candidate genes that may influence Cu accretion of sheep. Gene enrichment analysis indicated that enriched functions and involved genes regulate processes involving inflammation and cell death. This study reveals promising regions and genes under selection for further studies to investigate their influence on Cu accretion and potential use in sheep breeding.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101329.

Ethics approval

Not applicable.

Data and model availability statement

None of the data were deposited in an official repository. All data included in this study are available on request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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