Original research

PNPLA3 fatty liver allele was fixed in Neanderthals and segregates neutrally in humans

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ABSTRACT

Objective Fat deposition is modulated by environmental factors and genetic predisposition. Genome-wide association studies identified *PNPLA3* p.I148M (rs738409) as a common variant that increases risk of developing liver steatosis. When and how this variant evolved in humans has not been studied to date.

Design Here we analyse ancient DNA to track the history of this allele throughout human history. In total, 6444 published ancient (modern humans, Neanderthal, Denisovan) and 3943 published present day genomes were used for analysis after extracting genotype calls for *PNPLA3* p.I148M. To quantify changes through time, logistic and, by grouping individuals according to geography and age, linear regression analyses were performed.

Results We find that archaic human individuals (Neanderthal, Denisovan) exclusively carried a fixed *PNPLA3* risk allele, whereas allele frequencies in modern human populations range from very low in Africa to >50% in Mesoamerica. Over the last 15 000 years, distributions of ancestral and derived alleles roughly match the present day distribution. Logistic regression analyses did not yield signals of natural selection during the last 10 000 years.

Conclusion Archaic human individuals exclusively carried a fixed *PNPLA3* allele associated with fatty liver, whereas allele frequencies in modern human populations are variable even in the oldest samples. Our observation might underscore the advantage of fat storage in cold climate and particularly for Neanderthal under ice age conditions. The absent signals of natural selection during modern human history does not support the thrifty gene hypothesis in case of *PNPLA3* p.I148M.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hepatic fat deposition is modulated by genetic predisposition including *PNPLA3* p.I148M as a common variant that increases the risk of developing steatotic liver disease.
⇒ When and how this variant evolved in humans has not been studied to date.

WHAT THIS STUDY ADDS

⇒ The origin of the *PNPLA3* p.148M variant dates back beyond the split of the human lineages in evolution and was fixed in archaic humans like Neanderthal, putatively due to advantages in cold adaptation.
⇒ Within a time window of 10 000 years, distributions of ancestral and derived alleles roughly match the distribution we observe today.
⇒ No signal of natural selection in modern humans during the last 10 000 years or along latitude could be observed.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings in archaic humans support a putative advantage of genetic variants favouring intrahepatic fat storage in cold climate conditions.
⇒ Further extrahepatic functions of *PNPLA3* p.148M, for example, on thermogenesis, deserve future mechanistic investigation.

INTRODUCTION

The liver is the central organ in the human body responsible for carbohydrate and lipid metabolism. It plays a key role in fat storage under conditions of overnutrition. Fat deposition in the liver can range from benign forms of steatosis to inflammatory and progressive conditions, such as steatohepatitis (MASH) which are summarised as metabolic dysfunction-associated steatotic liver disease (MASLD). MASH can lead to liver fibrosis and hepatocellular carcinoma (HCC). The deposition of fat in the liver is mostly modulated by environmental factors, but also by genetic predisposition.[1] Studies in twins demonstrated that both NAFLD (now termed MASLD) and fibrosis are heritable traits (heritability 0.52).[2] Genome-wide association studies[3] have identified a common variant in the gene *PNPLA3* p.I148M as being prominently associated with increased risk of developing steatotic liver disease. The same gene modulates the risk of progressive inflammation (MASH) including its consequences, namely liver cirrhosis and HCC.[4] As much as 50% of adults with NAFLD (MASLD) carry at least one copy of the *PNPLA3* p.I148M risk allele (rs738409).[5] The extent of potentially harmful phenotypes (ie, end-stage liver diseases) associated with the *PNPLA3* variant suggests a potential effect on fitness and hence a potential role of natural selection. The risk variant of *PNPLA3* segregates in present-day human populations, ranging from 8% to 72%.[6]

The thrifty gene hypothesis represents an approach to explain why unfavourable genes

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Table 1 Genotypes for *PNPLA3* rs738409 in selected ancient hominins and Great Apes

Individual	Phylogeny	Country	Continent	Genotype
Orangutan (Reference)	Great Apes	n/a	n/a	C
Gorilla (Reference)	Great Apes	n/a	n/a	C
Chimpanzee (Reference)	Great Apes	n/a	n/a	C
Bonobo (Reference)	Great Apes	n/a	n/a	C
Altai_published.DG	Neanderthal	Russia	Asia	G/G
Mezmaiskaya1 _final_provisional.SG	Neanderthal	Russia	Asia	G/G
Chagyrskaya08.SG	Neanderthal	Russia	Asia	G/G
Goyet_final _provisional.SG	Neanderthal	Belgium	Europe	G/G
VindijaG1_final _provisional.SG	Neanderthal	Croatia	Europe	G/G
Chagyrskaya01	Neanderthal	Russia	Asia	G
Chagyrskaya02	Neanderthal	Russia	Asia	G
Chagyrskaya06	Neanderthal	Russia	Asia	Missing
Chagyrskaya07	Neanderthal	Russia	Asia	G
Chagyrskaya09	Neanderthal	Russia	Asia	Missing
Chagyrskaya11	Neanderthal	Russia	Asia	G
Chagyrskaya12	Neanderthal	Russia	Asia	G
Chagyrskaya13	Neanderthal	Russia	Asia	Missing
Chagyrskaya14	Neanderthal	Russia	Asia	Missing
Chagyrskaya17	Neanderthal	Russia	Asia	Missing
Chagyrskaya18	Neanderthal	Russia	Asia	G
Chagyrskaya19	Neanderthal	Russia	Asia	Missing
Chagyrskaya20	Neanderthal	Russia	Asia	G
Chagyrskaya41	Neanderthal	Russia	Asia	G
Chagyrskaya60	Neanderthal	Russia	Asia	G
Okladnikov11	Neanderthal	Russia	Asia	Missing
Denisova_published.DG	Denisovan	Russia	Asia	G/G
Denisova11.SG	Denisovan	Russia	Asia	G/G
GoyetQ116-1_published	Modern humans	Belgium	Europe	C
GoyetQ-2	Modern humans	Belgium	Europe	C
GoyetQ-2_udg	Modern humans	Belgium	Europe	C
GoyetQ116-1_udg_ published	Modern humans	Belgium	Europe	C
Ust_Ishim_published.DG	Modern humans	Russia	Asia	G/G
MA1.SG	Modern humans	Russia	Asia	G
Tianyuan	Modern humans	China	Asia	G
Sunghir2.SG	Modern humans	Russia	Asia	G
Sunghir3.SG	Modern humans	Russia	Asia	G
ZBC_IPB001.B-C0101 _Luk2-Pinarbasi	Modern humans	Turkey	Asia	G
Yana_old.SG	Modern humans	Russia	Asia	G
Pavlov1_d	Modern humans	Czech Republic	Europe	G
I2483	Modern humans	Austria	Europe	G
PM1	Modern humans	Romania	Europe	G
PM1_d	Modern humans	Romania	Europe	G
Kostenki14.SG	Modern humans	Russia	Asia	C
Kostenki14	Modern humans	Russia	Asia	C
Sunghir4.SG	Modern humans	Russia	Asia	C
Yana_old2.SG	Modern humans	Russia	Asia	C
NE20	Modern humans	China	Asia	C
NE56	Modern humans	China	Asia	C
Vestonice15_d	Modern humans	Czech Republic	Europe	C
Ostuni1_d	Modern humans	Italy	Europe	C
ElMiron_d	Modern humans	Spain	Europe	C

Single-copy alleles are from low-coverage data and pseudo-haploid (single-read). C—major allele, G—minor allele and associated with fatty liver.

involved in metabolism that evolved for survival in the late Palaeolithic era are frequent today. For example, the ability to store fat was likely an advantage throughout most of human history,[7] and as a consequence, mutations in genes facilitating fat storage were likely under positive natural selection and thus increased in frequency in the past. Indeed, obesity has likely been present in humans since the European upper Palaeolithic age.[8] Thrifty genes facilitate efficient fat storage when there are plenty of available foods and hold importance for reproduction under conditions of intermittent feast and famine.[7]

Advances in ancient DNA technology now enable studying natural selection by analysing samples from populations throughout time and space. The first genome-wide analysis of West Eurasians dating to between 6500 and 1000 before present (BP) revealed the strongest signal of selection for lactase persistence in Europe but independent signals in this specific population also included fatty acid desaturase genes (*FADS1* and *FADS2*) involved in lipid composition in human blood and tissue.[9]

To understand the past trajectory of the *PNPLA3* p.I148M variant throughout human evolution and history, we turn to archaeogenetic data, which is becoming increasingly available, with now more than 6000 published ancient genomes spanning the last 50 000 years[10] (although the vast majority fall within the last 10 000 years).

METHODS

Dataset

The dataset is based on the Allen Ancient DNA Resource (AADR)[10] version 50, using a version that had been curated via tools of the Poseidon Framework[11] (both the data and the tools are freely available, see Code and Data availability statement). The dataset consists of genetic data and spatiotemporal context data for thousands of samples worldwide. A list of primary publications underlying the AADR is found in online supplemental text 1. Temporal data comes in the form of date intervals (eg, 2000 BC–1500 BC), either derived from radiocarbon data, or from archaeological information of the burial site. For the analyses presented here, we chose the mid-point of each date interval for every sample. The spatial data comes in the form of a longitude and latitude for each sample. Finally, the genetic data consists of a matrix of N columns and L rows, where N ~10 000 is the number of ancient and modern samples, and L ~1.2 million is the number of single nucleotide polymorphic positions for which genetic data has been derived from the raw data. In this study, we extracted only the key single-nucleotide polymorphism (SNP) of interest (rs738409) and selected 1000 additional random SNPs using a custom computational pipeline in *bash* (see Code and Data Availability) and the Poseidon software trident.[11]

In the AADR, as common for low-coverage ancient genomic data, most samples are represented in so-called pseudo-haploid form, which means that a sample at a given SNP is represented by only a single allele (which in case of a heterozygote is a random choice of the two alleles). The resulting genotype is thus either of frequency 0 (homozygous-reference) or 1 (homozygous-alternative), and only in case of few ancient high-coverage genomes (eg, several of the archaic genomes[12 13]) and modern data can also be 0.5, which corresponds to a heterozygous genotype.

Regression analysis and selection tests

All analyses were performed in RStudio with R V.3.6.3 (2020-02-29). For the linear regression, individuals were combined

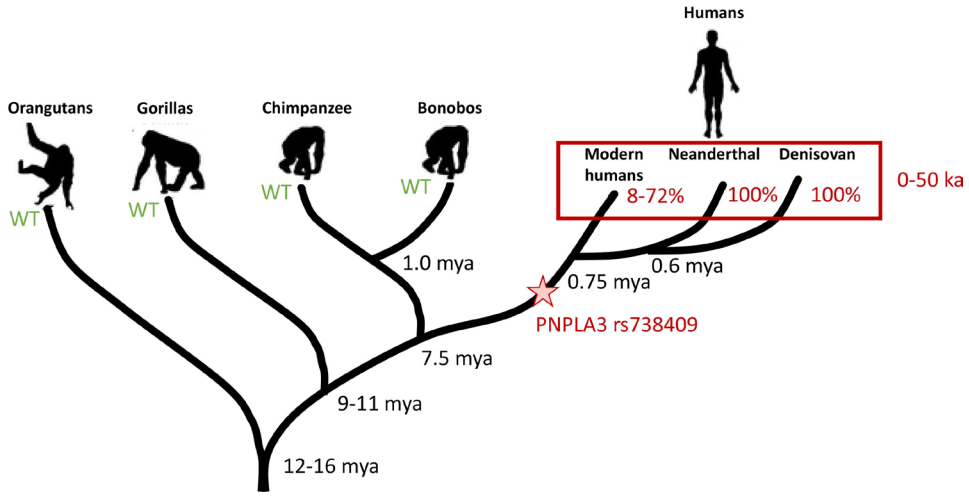

Figure 1 Presence of *PNPLA3* rs738409 major and minor alleles in the great ape lineage family tree. Established estimates of split times are indicated. The presumed time of the mutation underlying the variant of focus is indicated by a red-star. Allele frequencies of the minor allele in present-day and archaic humans are indicated. All great apes outside the human branch carry the wild type (WT) allele, as indicated. mya, million years.

into groups according to geography and age proximity (see online supplemental figure 1). The coefficients of the regression lines were calculated using the *lm* function of the inbuilt package *stats*. Corresponding straight lines were plotted with *geom_abline* function of the *ggplot2*-package of the *tidyverse* collection.

Since the *PNPLA3* variable is mostly categorical (except for a few high coverage archaic genomes listed in table 1 that were not included in the regression analyses), we examined whether the probability of carrying the minor allele is increasing or decreasing over time using logistic regression. First, the behaviour of the probabilities over time for each continent and some self-defined subregions was analysed on a visual basis. For this, the logistic regression curves were plotted by the *stat_smooth* function also from the package *ggplot2* package of *tidyverse*.

In a further step, we extended the logistic regression to a genome-wide analysis, using the age of the sample as the independent variable and the presence of the minor allele as the response (dependent) variable. We compared the absolute value of the β regression coefficient estimate, which yields the strength of correlation between age and response of the *PNPLA3* variant, with the same coefficient of 1000 randomly chosen genes as neutral control. The logistic fits were computed using the *glm* function of the inbuilt package stats with the input parameter family set to 'binomial'. In a similar fashion, we conducted logistic regressions with latitude as independent variable.

RESULTS

We explored the deeper evolutionary context of *PNPLA3* rs738409 in other species from the human lineage tree using reference genome sequences of current primates, and its more recent trajectory using dataset of 6444 published ancient and 3943 published present-day genomes,[10] and extracted genotype calls for the *PNPLA3* risk allele rs738409 (see Data and Code Availability below). The ancestral allele, which is also the reference allele (C), is fixed among primates, with all great apes (Chimpanzee, Bonobo, Gorilla, Orangutan) carrying the ancestral allele (figure 1). In contrast, all available Neanderthal (n=21) and Denisovan individuals (n=2, including a Neanderthal/Denisovan hybrid) either exclusively carried the risk allele (see table 1), or had missing data (n=7) suggesting fixation of the allele in the ancestor of all archaic humans. In contrast to archaic humans, present-day humans exhibit a wide range of minor allele frequency ranging from 8% in Kenya up to 72% in Peru.[6]

The presence of the reference allele (wildtype) in modern humans, despite the fixation in Neanderthals, poses the question of its trajectory. To investigate this, we first visually inspected the allele frequencies from present-day backwards through the last 15 000 years, where we have sufficient ancient genomes in most regions to estimate allele frequencies (figure 2). Overall, within this relatively recent window of the last 15 000 years, distributions of ancestral and derived alleles roughly match the distribution we observe today, including a high frequency in the Americas even in the earliest samples from South and North America from around 10 000 years ago.

To quantify changes through time, we performed two regression analyses in modern human populations: First, logistic regression based on presence/absence of the risk allele in ancient and modern individuals through time, separately for different continental groups; second, linear regression based on allele frequencies by grouping individuals according to geography and age (see the Methods section). As displayed in online supplemental figure 2A–D the frequency of the risk allele decreases over time from 15 000 BP to present, most prominently observed in Asia and America (although temporal coverage of ancient samples varies among continental groups), which is mirrored in the linear regression analysis (online supplemental figure 1A–D).

To compare these changes (negative slope of allele frequency over time with formal statistical significance for each continent, $p<0.05$) with expected changes due to neutral factors such as genetic drift, we compiled a reference dataset of 1000 randomly selected SNPs within the published genotyping data, and performed the same logistic regression on each of these randomly selected SNPs (figure 3). On all continents, the observed frequency change of the *PNPLA3* p.I148M SNP fell inside the distribution, within two SD, of expected frequency changes as estimated from the neutral reference SNP set. We quantified this further by computing the fraction of SNPs with absolute regression coefficients larger than that of *PNPLA3*, resulting in p values of 0.15, 0.07, 0.30 and 0.62 for the four regional cases displayed in figure 3. Hence, the data does not

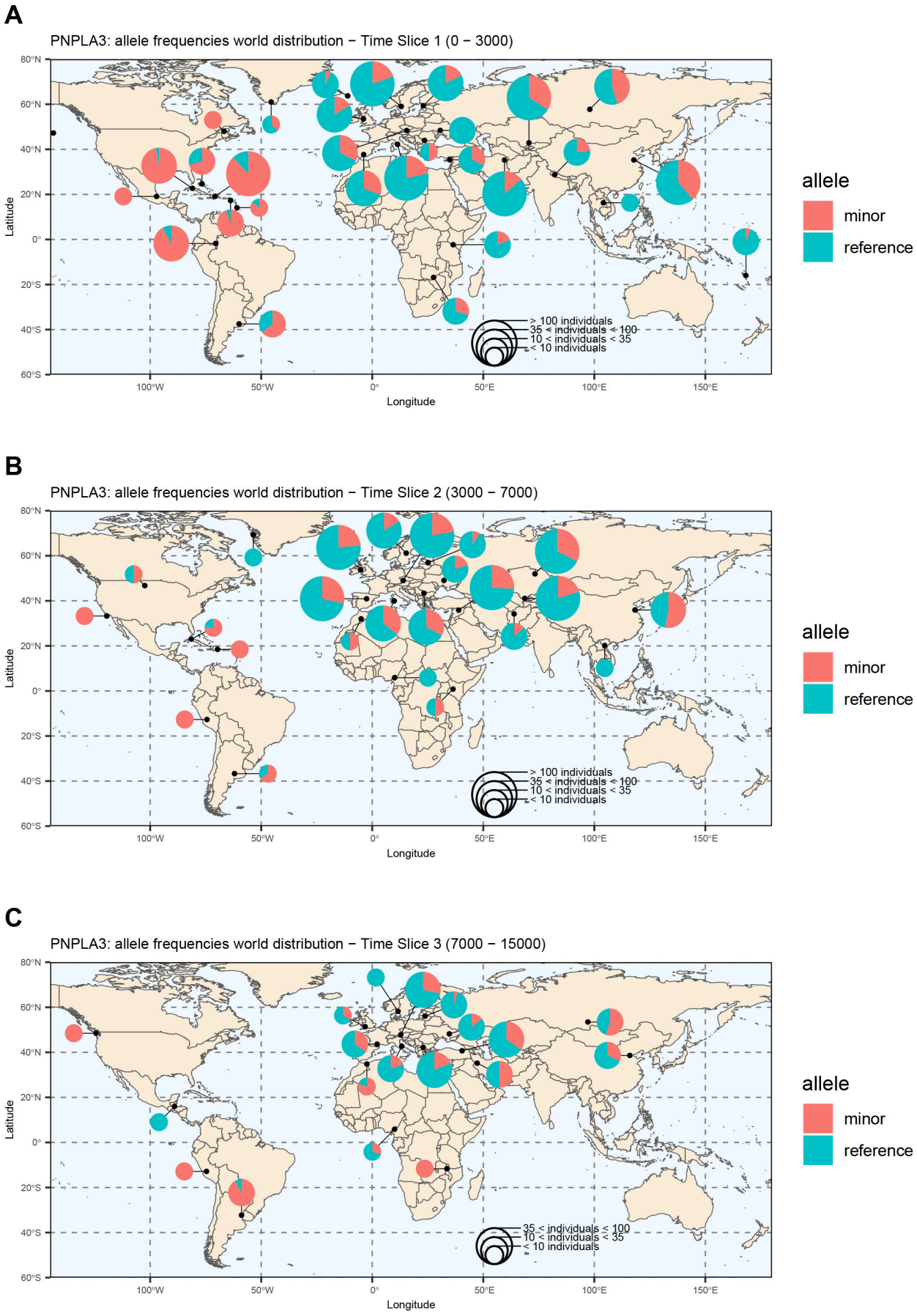

Figure 2 Allele frequencies of the *PNPLA3* minor risk allele (rs738409) in three time slices as observed from ancient DNA. Individuals were grouped by time and geography (see online supplemental figure 2 for groupings). Sample sizes are indicated by sizes of pie-charts, as indicated in the legend.

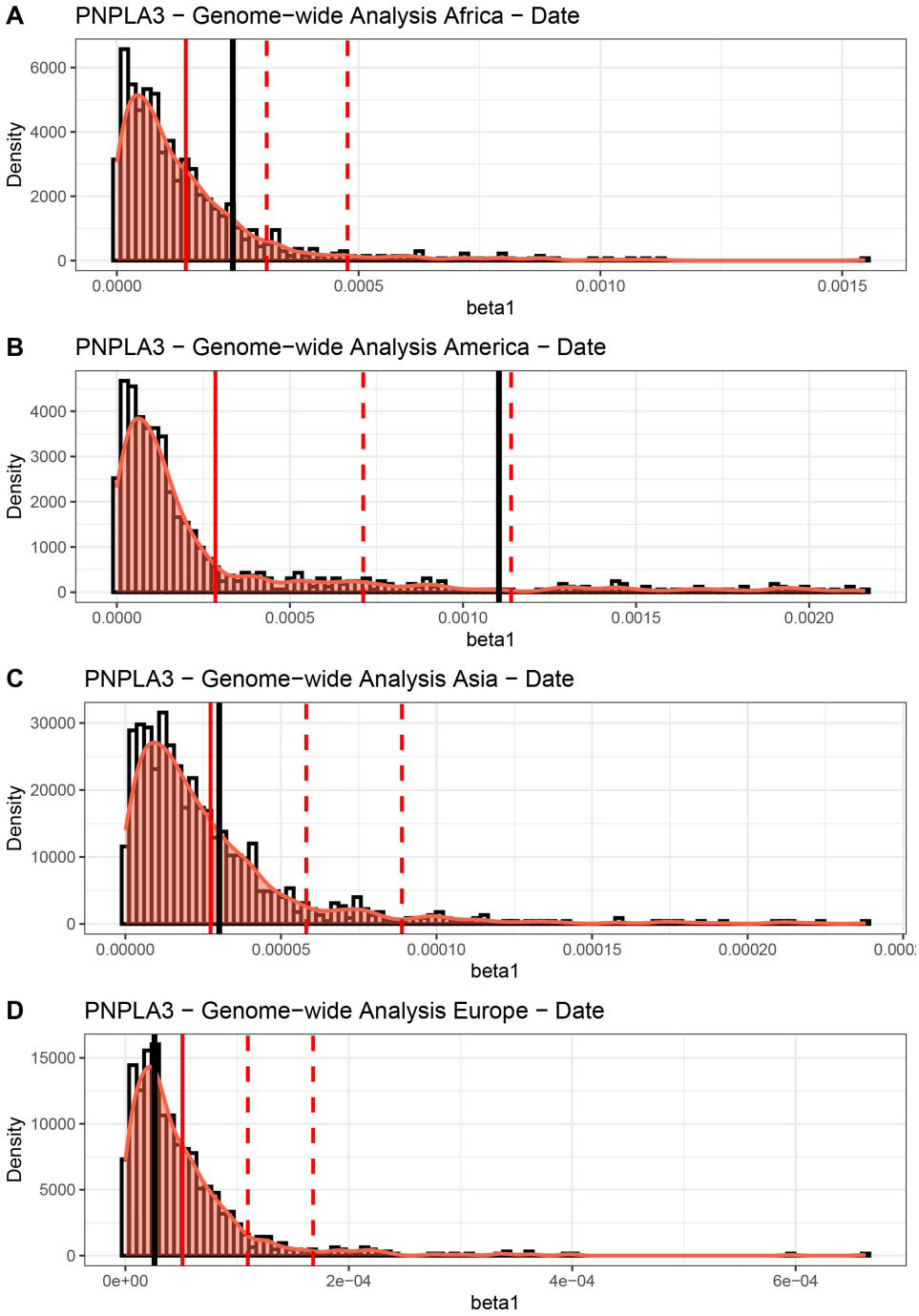

Figure 3 Genome-wide analysis of logistic regression coefficients using 1000 randomly selected single-nucleotide polymorphisms, in comparison to the result from the *PNPLA3* risk allele, with respect to sample date. The x-axis denotes the absolute value of the regression coefficient. The red solid line indicates the mean of the distribution, the two dashed red lines mark one and two SD, respectively. The black solid line indicates the result for the *PNPLA3* risk allele. Note the different scales of the x-axis.

support a significant contribution of natural selection to explain the observed changes in allele frequency over time in the different continents, even though the generally observed decrease in frequency may still be suggestive of moderate selection.

Given the fact of high allele frequency in the Americas, we considered whether there might be an effect on fitness in higher latitudes (given that humans settled in the Americas from Siberia). We therefore also conducted a logistic regression with formally significant changes along latitude ($p<0.05$) (online supplemental figure 3), and again compared with our randomly selected reference SNP set (online supplemental figure 4). Again, the observed changes in modern human populations were within the expected variation, consistent with absence of natural selection either through time or along latitude (corresponding to non-significant p values of 0.23, 0.12, 0.18 and 0.17 for the four cases displayed in online supplemental figure 4).

DISCUSSION

The most surprising aspect of our analysis is the fact that archaic human individuals (Neanderthals and Denisovans) exclusively carried a fixed *PNPLA3* rs738409 fatty liver allele, whereas

allele frequencies in modern human population range from very low in African populations to more than 50% in Mesoamerica. The presence of variant *PNPLA3* in African populations argues against introgression from Neandertals into modern humans as the main source of this variant in contemporary populations although it may have contributed to it. Since the *PNPLA3* p.I148M minor allele is present both among Neandertals and modern humans, this polymorphism is likely older than the split between Neandertals and modern humans.

While our genome-wide analysis did not detect significant signals of natural selection during modern human history throughout the last 10 000 years and by latitude, there is still the possibility of selection being active in time periods older than what we can statistically analyse using archaeogenetic time-series data of allele frequencies.

Liver, where PNPLA3 is primarily expressed[14] and where it exerts its functions, might be seen as one of the fat storing organs in humans. Indeed, fat storage in the liver confers physiological functions for times of starvation in various species, for example, in ducks before setting out for long distance flights.[15] PNPLA3 is also highly expressed in the retina and is involved in the metabolism of vitamin A.[16] One can speculate that our observation underscores the advantage of fat storage in cold climate and particularly for Neanderthals under ice age conditions. Supportive of this hypothesis, the *PNPLA3* rs738409 risk allele is predominant in 89.3% of the Yakut population in the coldest northeast region of Russia.[17]

Fat storage and mobilisation in adipose tissue and liver are critical processes for systemic energy homeostasis that involve dynamic interactions among evolutionarily conserved proteins including members of the Patatin family of lipases. Enhanced indirect suppression of lipolysis, combined with loss of intrinsic lipase activity by *PNPLA3* p.I148M expression likely explains a pronounced accumulation of triacylglycerol in brown adipose tissue.[18] This is of functional relevance for cold adaptation since brown adipose tissue drives non-shivering thermogenesis which represents a central mechanism of enhancing the energy expenditure for heat production through the actions of uncoupling protein 1.[19]

As another gene locus favouring hepatic fat storage, a variant in the *SLC16A11* gene predisposing to diabetes mellitus nowadays, has been shown to have been introduced to modern humans from Neanderthals.[20] In line with timely expedited fat storage on availability, a feed-forward activation loop of *PNPLA3* expression in the liver has been observed.[14]

According to consistent pathological skeletal changes in one *Homo erectus* fossil ancient populations may have been at risk of vitamin A hypervitaminosis.[21] This disorder can be attributed to the high dietary intake of animal liver, most probably that of carnivores. Particularly for arctic environments, it has been shown that the liver of top predators contained about 10–20 times more vitamin A than the liver of other arctic and continental animals.[22] Of note, vitamin A intoxication has even been noticed after ingestion of large carnivorous fish liver in contemporary Europe.[23] The minor *PNPLA3* p.I148M allele might have modulated the metabolism of vitamin A and lowered the toxic effects of high vitamin A concentrations in the circulation. Indeed, carriers of the *PNPLA3* p.148M allele show lower fasting circulating retinol concentrations.[24] Since circulating retinol levels under fasting conditions are a function of retinol mobilisation from cellular sources as shown for hepatic stellate cells, variant *PNPLA3* p.148M protein putatively induces an intracellular retention due to a loss of retinyl-palmitate esterase activity.[16]

Whether intracellular retention of retinol conferred by variant *PNPLA3* p.148M protein contributes to visual performance of the retina is unknown to date. However, it is tempting to speculate that variant *PNPLA3* might have impacted activities of Neanderthal individuals dealing with less light and especially dim winters in higher latitudes of Eurasia. Better vision in low-light conditions might have usefully extended the day. Given the limited lifespan of archaic and early modern humans, it is at least not surprising that no signal towards negative selection can be detected, since arguably this variant mostly exerts its unfavourable effects rather in later adult life[5] and hence is less likely to impact reproductive dynamics (fitness).

Despite several putative advantages of the variant *PNPLA3* allele during most of the time in human history, the thrifty gene hypothesis, an approach to explain why unfavourable genes involved in metabolism are frequent today, seems not to be a valid explanation in case of variant *PNPLA3* p.148M, at least not within our scope of analysis, which is restricted to the last 15 000 years.

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Contributors AG, MK and SS conceived and designed the study. JT, KW, CS and SS performed bioinformatic data preparation. JT performed analysis and prepared figures. AG, MK and SS wrote the paper with input from all coauthors. AG and SS act as the guarantors of the study.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. All data and code for analysis are provided in a github repository: https://github.com/stschiff/PNPLA3_AADR_analysis. Raw data is from the Allen Ancient DNA Resource version 50. A reference list of all 172 publications that report primary data is available as online supplemental text file 1.

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