Changes in Biological Pathways During 6,000 Years of Civilization in Europe

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Abstract

The beginning of civilization was a turning point in human evolution. With increasing separation from the natural environment, mankind stimulated new adaptive reactions in response to new environmental factors. In this paper, we describe direct signs of these reactions in the European population during the past 6,000 years. By comparing whole-genome data between Late Neolithic/Bronze Age individuals and modern Europeans, we revealed biological pathways that are significantly differently enriched in nonsynonymous single nucleotide polymorphisms in these two groups and which therefore could be shaped by cultural practices during the past six millennia. They include metabolic transformations, immune response, signal transduction, physical activity, sensory perception, reproduction, and cognitive functions. We demonstrated that these processes were influenced by different types of natural selection. We believe that our study opens new perspectives for more detailed investigations about when and how civilization has been modifying human genomes.

Key words: **microevolution, selection, European civilization, adaptation, ancient DNA, pathway analysis.**

Introduction

It is generally accepted that the term "civilization" refers to any complex society characterized by urban development, social stratification, symbolic communication forms (typically represented by writing systems), and a perceived separation from and domination over the natural environment (Adams 1966). From an evolutionary point of view, civilization started when humans, instead of reacting to the environment, began to actively shape it. Since the Neolithic transition, mankind has experienced a shift to agriculture, domestication of animals and plants, sedentism, significant increase in population density, and exposure to new pathogens; most of these effects have been self-imposed. Humans have been creating the artificial environment separating them from nature. This new environment induces new responses to it.

At present, it is supposed that culturally derived selection pressures should be stronger than noncultural ones (Feldman and Laland 1996; Ehrlich 2000; Bersaglieri et al. 2004; Richerson and Boyd 2005; Laland 2008; Laland et al. 2010). The main reason for this is that using cultural practices led to drastic population growth. As a result, the number of targets for mutations (both advantageous and disadvantageous) in the population increased, as did the number of individuals for selection (Laland 2008). Paradoxically, mutations accumulated in human genomes as a result of relaxed natural selection can also serve as targets for selection in new environmental conditions. Moreover, new cultural practices typically spread more quickly than genetic mutations, and the more individuals exhibiting the cultural trait, the greater the intensity of selection (Kimura 1955; Boyd and Richerson 1985; Hawks et al. 2007; Laland 2008; Cochran and Harpending 2009).

Culturally derived selection leaves signs in the human genome. Some of these signs (like lactase persistence) are quite evident (Holden and Mace 1997; Beja-Pereira et al. 2003; Gamba et al. 2014; Allentoft et al. 2015), whereas many others are still uncertain (Libert et al. 1998; Stephens et al. 1998; Galvani and Slatkin 2003; Sabeti et al. 2005). Revealing and analyzing these selection signatures is of high importance not only for improving our understanding of connections between the human organism and the environment but also for deepening our insight into mechanisms of emergence of the so-called "diseases of civilization."

Article

The earliest stages of human civilization are the Late Neolithic Age and the Bronze Age. These were the epochs that gave rise to our present lifestyle. The 6,000 years between that period and modern times encompass the greater part of human civilization events. In this paper, we study the genetic consequences of these cultural events.

Many different approaches are used to reveal and analyze possible selection signals (Voight et al. 2006; Sabeti et al. 2007; Tang et al. 2007; Williamson et al. 2007; Quach et al. 2009; Grossman et al. 2013; Mathieson et al. 2015; Field et al. 2016). Most are based on modern human genome-wide data and, therefore, represent indirect evidence of selection. Objective information can be obtained by direct comparison of ancient and modern human genomes. The first steps in this direction were made relatively recently; they became possible thanks to whole-genome next generation sequencing of ancient samples. These studies have revealed selection signatures in single nucleotide polymorphisms (SNPs) associated with skin pigmentation, diet, and immunity, as well as with some complex traits, that is, human height (Olalde et al. 2014; Allentoft et al. 2015; Mathieson et al. 2015; Dannemann et al. 2016; Fu et al. 2016).

Providing that natural selection should act through phenotypes, we assume that selection signals for multigenic traits should be analyzed not only at the level of individual SNPs but also at the level of biological pathways, where the influence of individual SNPs is aggregated into functional groups. This approach has been previously used, for instance, to study selection signatures between human and chimpanzee lineages (Somel et al. 2013). In the present study, we applied pathway analysis to low-covered whole-genome ancient DNA sequence data. We compared data on European Late Neolithic/Bronze Age individuals (Gamba et al. 2014; Allentoft et al. 2015; Haak et al. 2015; Mathieson et al. 2015) with those from modern European individuals (http://www.internationalgenome.org/) supposedly of Bronze Age ancestry and occupying the same geographical area as their ancestors. Our aims were 1) to reveal nonsynonymous SNPs in ancient and modern groups, 2) to associate these SNPs with biological pathways, and 3) to calculate the differences in pathway enrichment between the ancient and modern groups. The revealed differences indicate the processes that we suppose have been shaped by introduction of human cultural practices during the past 6,000 years.

Results

Compatibility of the Data

We compared whole-genome data from 150 ancient samples (supplementary tables 1 and 2, Supplementary Material online) dated between 3,500 and 1,000 BCE (Gamba et al. 2014; Allentoft et al. 2015; Haak et al. 2015; Mathieson et al. 2015) (fig. 1) with data on 305 modern Europeans genotyped in the framework of the 1000 Genomes Project (Gibbs et al. 2015). We analyzed 40,573 synonymous and 48,860 nonsynonymous SNPs from the Bronze Age group versus 72,558 synonymous and 96,710 nonsynonymous SNPs from the modern group using the pipeline shown in figure 2.

To test whether there is any genetic continuity between the Bronze Age group and the modern group, we applied two different approaches. First, principal components analysis (PCA) demonstrated that the ancient and modern European individuals are colocated within the same cluster and are separated from modern individuals from other geographic regions (Africa, America, and Asia) (fig. 3).

Second, to test whether the analyzed modern individuals possess genetic ancestry of the Bronze Age individuals, we measured the proportion of the Bronze Age individuals in modern samples. Figure 4 shows that the linear composition of Bronze Age ancestry in the modern individuals is relatively high and varies from 20% to 90%.

Therefore, we can confidently consider the analyzed modern Europeans to be genetic descendants of the Bronze Age Europeans; this fact gives us the basis for studying microevolution changes that occurred during the past six millennia in Europe.

Comparison of Ancient and Modern Data

Due to the low coverage of each position on the genome in the ancient data, consideration of individual SNPs for direct comparison of ancient and modern data does not produce biologically or statistically significant results, since variant call at each ancient genomic position has limited fidelity. Therefore, we considered one ancient merged genome and one modern merged genome. The ancient merged genome was assembled from compiling all SNPs of European Bronze Age individuals, whereas the modern merged genome was assembled from all SNPs of modern European individuals. Grouping of the SNPs into KEGG biochemical pathways (see Materials and Methods) gave the additional robustness to the calculations.

We assumed that during neutral evolution the same biological pathways in the ancient and in the modern groups should accumulate mutations at the same rate, whereas under selection pressure the rate of accumulation of mutations in the same pathways should be different. Therefore, we calculated two types of enrichment scores for pathways: 1) differential synonymous SNP enrichment (DSSE) scores between ancient and modern groups and 2) differential nonsynonymous SNP enrichment (DNSE) scores for these groups (see Materials and Methods). The enrichment score for each pathway was calculated as the deviation of the fraction of ancient SNPs in the given pathway from the expected fraction of SNPs in the ancient merged genome. Therefore, when there are more SNPs in the ancient merged genome, compared with what is expected, the enrichment score is positive; when there are less SNPs in the ancient merged genome, compared with what is expected, the enrichment score is negative. Hence, a positive enrichment score indicates higher pathway enrichment in the ancient group; a negative enrichment score indicates higher pathway enrichment in the modern group.

Comparative analysis of DSSE scores revealed that none of the pathways show significant differences in synonymous SNP enrichment between the ancient and the modern groups (supplementary table 3, Supplementary Material online).

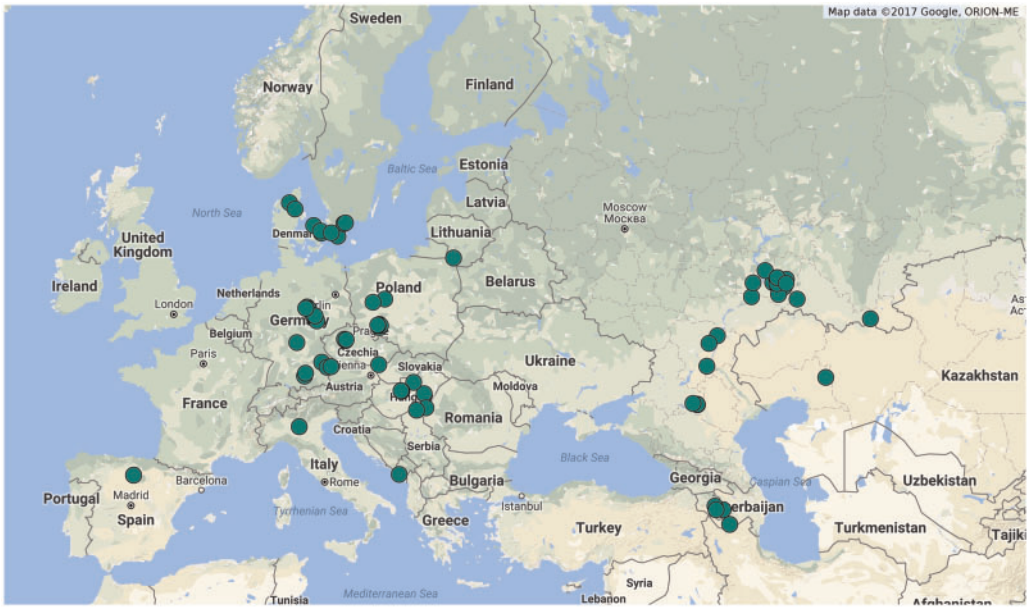

FIG. 1. Location of ancient samples analyzed in the study. Data from Allentoft et al. (2015), Gamba et al. (2014), Haak et al. (2015), and Mathieson et al. (2015) (for details, see supplementary tables 1 and 2, Supplementary Material online).

This corresponds to the hypothesis of neutral evolution for this type of mutations. At the same time, comparison of DNSE scores revealed 15 pathways that were differentially enriched in nonsynonymous SNPs between the Bronze Age and modern European individuals (fig. 5 and table 1; supplementary tables 4 and 5, Supplementary Material online). We also normalized nonsynonymous SNPs on synonymous SNPs. The results (fig. 6) showed that all *P*-values of the synonymous test, as well as most *P*-values of the nonsynonymous test are inside the area of nonsignificant differences (shaded rectangle). At the same time, *P*-values of the nonsynonymous test for 15 differently enriched pathways are outside the area of nonsignificant differences. This confirms the significance of differences in these pathways between ancient and modern Europeans.

The significance of the differences of enrichment scores between the ancient and the modern groups was assessed using the Bonferroni correction with $P < 0.01$ (table 1). Benjamin et al. (2017) proposed to use the threshold of $P < 0.005$ (see Materials and Methods). We suggest that, among 15 revealed pathways, the 2 pathways that did not pass this threshold (pentose and glucuronate interconversions and PI3K-Akt signaling pathway) should be interpreted with caution. We also excluded two of the pathways: metabolic pathways since this grouping is too general and ascorbate and aldarate metabolism since it is very reduced in humans and its functions are not unique (Ye and Doak 2009).

Therefore, we have identified the following pathways to be significantly different between the Bronze Age and modern groups: pentose and glucuronate interconversions, drug metabolism by cytochrome P450, chemical carcinogenesis, ABC transporters, antigen processing and presentation, graft-versus-host disease, autoimmune thyroid disease, hypertrophic cardiomyopathy, olfactory transduction, oocyte meiosis, long-term potentiation, and dopaminergic synapse.

We also compared the distribution of regulatory SNPs between Bronze Age and modern individuals (see Materials and Methods). A proportion test revealed no difference in enrichment of the KEGG pathways between the ancient and the modern groups. This result was expected since the functions of most of the revealed SNPs in the regulatory regions are not yet known. Nonfunctional SNPs contribute to noise interfering the detection of functional SNPs (the same situation could happen if we analyzed synonymous and nonsynonymous SNPs together).

Verification of the Results

As an alternative hypothesis, we considered the possibility that the obtained results can be explained by insufficient sequence coverage of pathways in Bronze Age individuals. To test this hypothesis, we performed the following computations. First, we calculated the Spearman correlation coefficient between enrichment score and fraction of covered length (supplementary fig. 1, Supplementary Material online). The coefficient of determination was $R^2 = 0.1$. This implies that a change in the coverage can explain only 10% of the variability of pathway SNP enrichment and cannot be the leading cause of the observed effect. Second, we analyzed the median coverage and median length of genes in the pathways (supplementary fig. 2, Supplementary Material online). With the rare exception (three pathways), more than 50% of individual genes were covered in the studied pathways. The revealed enriched pathways were clustered together with unenriched pathways. Third, we calculated average coverage per base pair per sample per pathway and total coverage per base pair per pathway (supplementary fig. 3, Supplementary Material online). In general, there is no relationship between enrichment and coverage. The only exception is olfactory

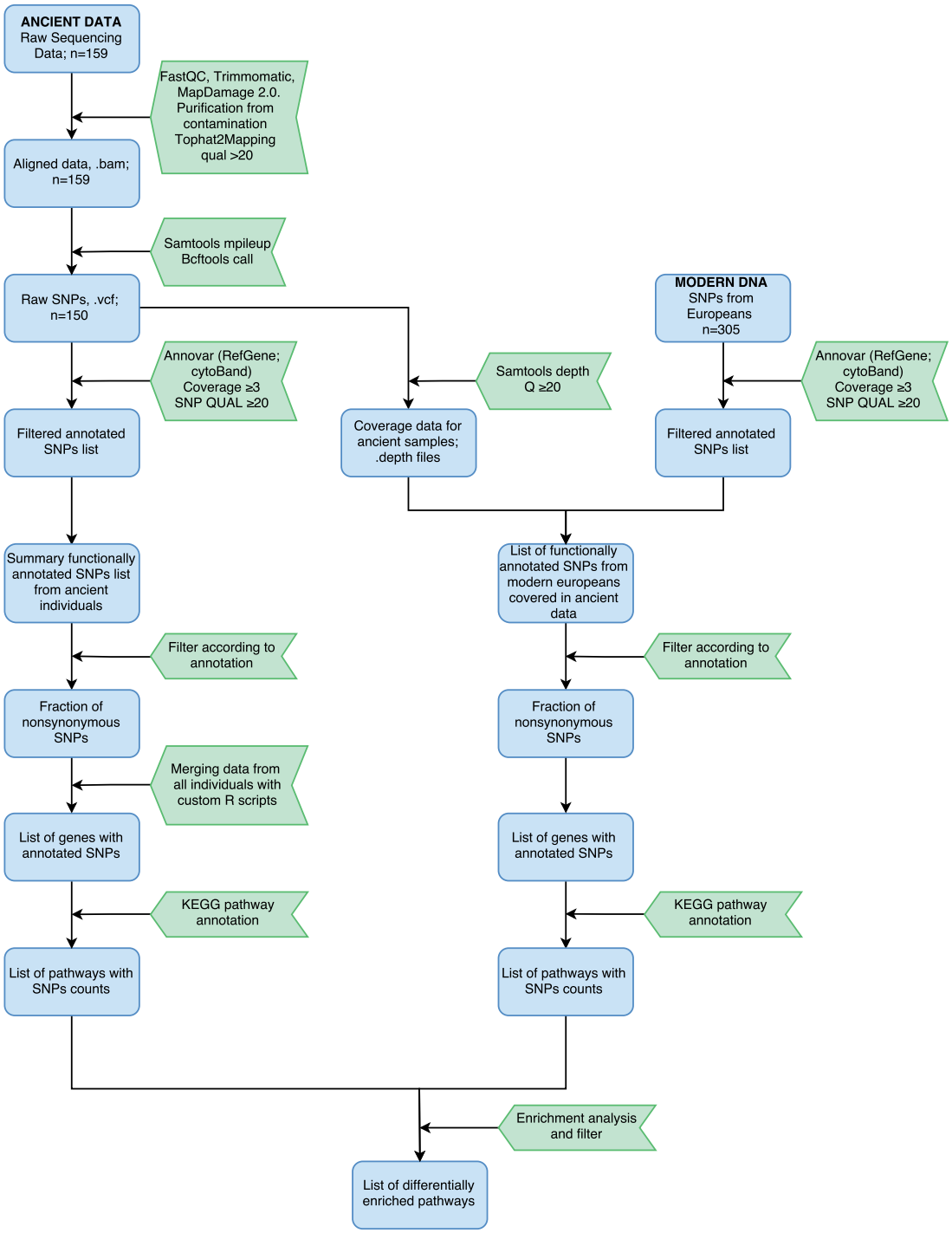

Fig. 2. Principal pipeline of the study. Analysis for nonsynonymous SNPs is presented. All calculations for synonymous SNPs were performed in the same manner. Additional parameters and tool versions are listed in supplementary methods, Supplementary Material online.

transduction pathway (supplementary fig. 3*A*, Supplementary Material online), whose average coverage per sample is a bit lower in comparison to other pathways. However, the total coverage for this pathway (supplementary fig. 3*B*, Supplementary Material online), though a bit lower in comparison to most of other pathways, is not an outlier (there are two other pathways with the same coverage which did not show any difference in enrichment between ancient and modern groups). For our calculations, we used data from total, not average, coverage. Therefore, there is no relationship between enrichment and the gene's size or coverage.

The observed trend might also be the result of general interpopulation differences between the two groups. To test this hypothesis, we calculated interpopulation differences between modern European groups using the same pathway enrichment analysis (supplementary table 6, Supplementary Material online). No difference in enrichment in any pathway was revealed between present-day Europeans. Therefore, the

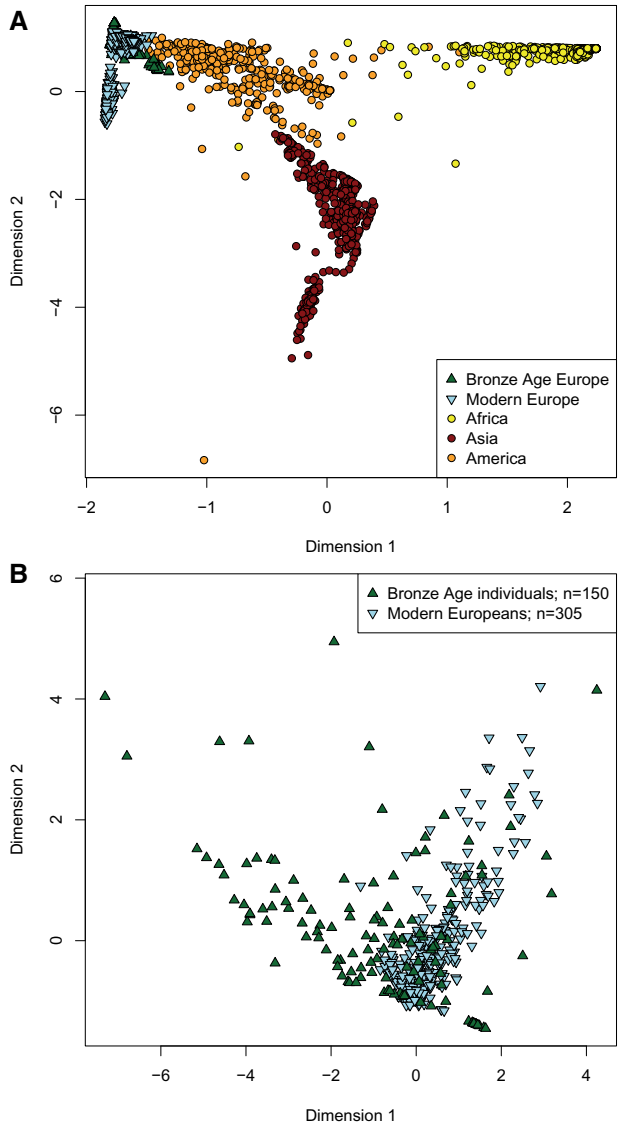

FIG. 3. Principal component analysis. (*A*) World groups and (*B*) European groups.

observed differences between the Bronze Age and modern groups are the results of microevolution changes during the past 6,000 years.

Discussion

Since the Late Neolithic, the European lifestyle has changed drastically. The main factors determining the relationship between environment and the human body have undergone significant alterations. For example, preagrarian and early agrarian populations were exposed to environmental influences from a comparatively small geographical area (Gillings et al. 2015). In contrast, modern Europeans exist in a globalized world where global travel (and corresponding environmental exposures) as well as different new types of food, clothes, and other consumables are common. Many new factors have appeared, such as dietary changes, new pathogens, new medications, as well as high population density and closer connections between distant groups of people. All of

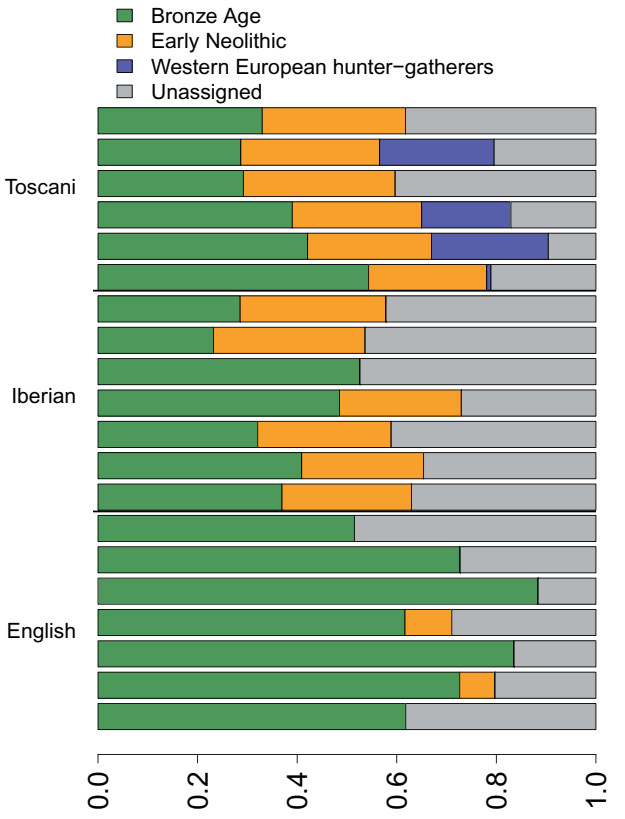

FIG. 4. Proportion of ancient genomes in modern European individuals.

these new conditions inevitably provoke responses from the human body.

In this paper, we studied how introduction of different cultural practices during the past 6,000 years could shape human genomes. We traced the microevolution of modern Europeans back to their ancestors, carriers of the Late Neolithic and Bronze Age cultures. We revealed 13 biological pathways that are significantly different between the Bronze Age and modern groups. For most of them (except 3) the number of nonsynonymous mutations is higher in the modern group than in the Bronze Age group, which means the accumulation of mutations during the past 6,000 years. In the next paragraphs, we attempt to explain what civilization events during the past millennia could have caused the changes in these pathways.

We detected significant changes in a number of pathways responsible for metabolism. One of them, pentose and glucuronate interconversions, is associated with carbohydrate metabolism. In the human organism, this pathway mainly describes the transformation of UDP-glucose, α-D-glucose-1-phosphate, and D-xylose (Du et al. 2016). We suggest that changes in this pathway are the consequences of dramatic diet modifications arising with the introduction of agriculture, an important event that stimulated the Neolithic transition and progressed during the Bronze Age. One of three substrates entering the pentose and glucuronate interconversions pathway, UDP-glucose, comes from the galactose metabolism pathway (Du et al. 2016). The main source of galactose in the modern human diet is lactose from milk.

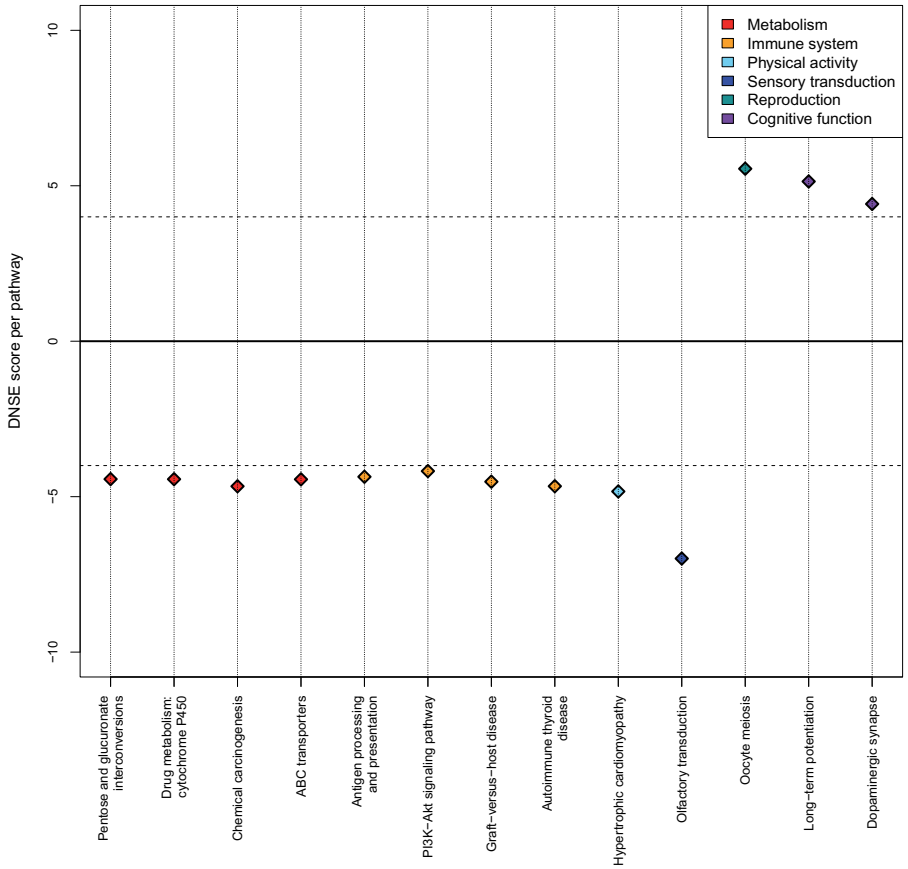

Fig. 5. Differential SNP enrichment of KEGG pathways between ancient and modern individuals. Positive DNSE values correspond to pathways that have more SNPs in genomes of ancient individuals, whereas negative DNSE values correspond to pathways that have more SNPs in genomes of modern Europeans.

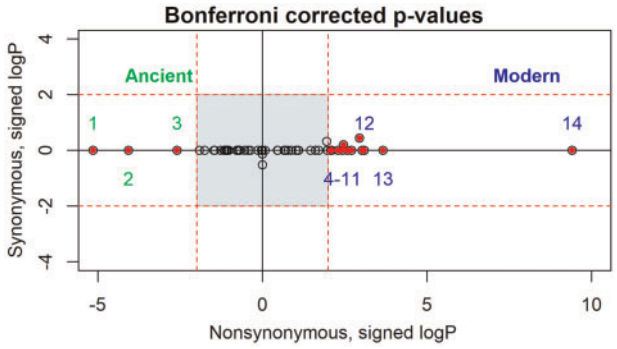

Fig. 6. Relationship between the *P*-values for synonymous and nonsynonymous SNPs in the studied pathways.

Humans are the only mammals who have the ability to utilize lactose in adulthood. This ability is provided by a single mutation in an enhancer region of the lactase gene (*LCT*) whose product lactase, a participant in the galactose metabolism pathway, breaks down lactose (Lewinsky et al. 2005; Enattah et al. 2008). It is believed that in Europe the *LCT* mutation arose in the Bronze Age or somewhat earlier as a result of milking (Holden and Mace 1997; Gamba et al. 2014; Allentoft et al. 2015). In modern Europe, the mutation frequency is up to 100% (Gerbault et al. 2011) indicating strong positive selection of this gene. Apparently, such a significant change in the galactose metabolism pathway could strongly affect the product (UDP-glucose) yield, which in turn could modify the next pathway, pentose and glucuronate interconversions. Other substrates for the pentose and glucuronate interconversions pathway are α-D-glucose-1-phosphate, the product of glycolysis, and D-xylose, entering from the starch and sucrose metabolism pathway (Du et al. 2016). Glucose and starch dairy intake has changed dramatically during the past 6,000 years: As a result of agriculture, the ratio of carbohydrate-rich food, especially grain-based products, has increased significantly in the human diet (Cordain et al. 2005). This ratio further increased after the Industrial transition in the 18–19th century after which industrially processed flour and sugar became commonly available (Cordain et al. 2005; Adler et al. 2013). Therefore, we suppose that changes in nutrient consumption and thus in the metabolism of substrates for the pentose and glucuronate interconversions pathway have caused an accumulation of nonsynonymous mutations, which could modify this pathway.

Other metabolic pathways are associated with the transformation of xenobiotics. They include drug metabolism by cytochrome P450 and chemical carcinogenesis (two closely related pathways, metabolism of xenobiotics by cytochrome P450 and drug metabolism by other enzymes, have passed only the Benjamini–Hochberg correction and not the Bonferroni one (supplementary tables 3 and 4,

Table 1. Biological Pathways Differently Enriched in Ancient and Modern Groups.

Pathway ID	Pathway Name	Ancient SNPs Count	Modern SNPs Count	DNSE Score	*P*-value	*P*-value Adjusted Bonferroni	Enriched Bonferroni 0.01 Threshold	Enriched Bonferroni 0.005 Threshold
hsa00040	Pentose and glucuronate interconversions	26	117	−4.29	1.75×10^{-05}	5.05×10^{-03}	Modern	No
hsa00053	Ascorbate and aldarate metabolism	20	99	−4.20	2.69×10^{-05}	7.77×10^{-03}	Modern	No
hsa00982	Drug metabolism—cytochrome P450	84	259	−4.38	1.20×10^{-05}	3.48×10^{-03}	Modern	Modern
hsa01100	Metabolic pathways	2512	4982	−4.69	2.76×10^{-06}	7.98×10^{-04}	Modern	Modern
hsa02010	ABC transporters	209	533	−4.44	8.93×10^{-06}	2.58×10^{-03}	Modern	Modern
hsa04114	Oocyte meiosis	298	337	5.58	2.41×10^{-08}	6.97×10^{-06}	Ancient	Ancient
hsa04151	PI3K-Akt signaling pathway	969	2019	−4.18	2.94×10^{-05}	8.50×10^{-03}	Modern	No
hsa04612	Antigen processing and presentation	232	578	−4.37	1.27×10^{-05}	3.66×10^{-03}	Modern	Modern
hsa04720	Long-term potentiation	202	215	5.13	2.91×10^{-07}	8.41×10^{-05}	Ancient	Ancient
hsa04728	Dopaminergic synapse	294	365	4.45	8.61×10^{-06}	2.49×10^{-03}	Ancient	Ancient
hsa04740	Olfactory transduction	756	1817	−7.09	1.35×10^{-12}	3.89×10^{-10}	Modern	Modern
hsa05204	Chemical carcinogenesis	101	305	−4.62	3.86×10^{-06}	1.12×10^{-03}	Modern	Modern
hsa05320	Autoimmune thyroid disease	181	482	−4.65	3.25×10^{-06}	9.38×10^{-04}	Modern	Modern
hsa05332	Graft-versus-host disease	166	444	−4.50	6.71×10^{-06}	1.94×10^{-03}	Modern	Modern
hsa05410	Hypertrophic cardiomyopathy (HCM)	347	843	−4.95	7.48×10^{-07}	2.16×10^{-04}	Modern	Modern

NOTE.—Differential SNP enrichment of KEGG pathways between ancient and modern individuals. Positive DNSE values correspond to pathways that have more SNPs in genomes of ancient individuals, whereas negative DNSE values correspond to pathways that have more SNPs in genomes of modern Europeans.

Supplementary Material online). These pathways are closely connected because they have partially overlapping mechanisms (Lang and Pelkonen 1999; Oliveira et al. 2007) (indeed, the chemical carcinogenesis pathway shares approximately 70% of genes with the cytochrome P450 metabolic pathway [supplementary table 7, Supplementary Material online]). During the past several millennia, substantial changes in human lifestyle were accompanied by the introduction of large amounts of different xenobiotics (including new types of food, alcoholic beverages, and microbial toxins). Some of them (such as medications, plant fertilizers, and food additives) are supposed to improve the quality of human life. Others (such as heavy metals and other pollutants) are side effects of civilization activities. All of these substances can shape human genomes by causing mutations (directly or indirectly) or by inducing natural selection. Our results suggest that new environmental factors in the form of xenobiotics have induced genomic responses via increasing gene variability and, as a result, modification of corresponding pathways. Unfortunately, we can see not only this adaptation but also an increase in the number of mutations in the chemical carcinogenesis pathway.

The ABC transporters pathway can be considered a part of the human metabolic system. Human ABC transporter genes encode transmembrane pumps that transport various substrates (including amino acids, lipids, proteins, inorganic ions, drugs, and other xenobiotics) against concentration gradients (Stefkova et al. 2004; Pohl et al. 2005; Vasiliou et al. 2009; Moitra and Dean 2011). Therefore, changes in the quantity or quality of these substrates through diet modifications or introduction of xenobiotics could also affect genes encoding these transport proteins. Interestingly, signals of positive selection were detected earlier in some genes associated with transport of vitamins and cofactors (Voight et al. 2006; Tang et al. 2007). In aggregate, these data suggest that changes in lifestyle have induced genetic modifications in a system for transport of nutrients and xenobiotics in the human body during the past several millennia.

Antigen processing and presentation is a very important part of the adaptive immune system, which is evolutionarily young and very reactive to environmental factors. It is the first line of host immune defense that recognizes and initiates immune responses to a broad range of alien agents. The major histocompatibility complex (MHC) plays the most important role in this process. Due to a very specific mechanism of antigen interaction, MHC proteins are highly diverse, and the genes encoding them (human leukocyte antigen genes, *HLA*) are the fastest evolving genes in the human body (Blum et al. 2013; Forni et al. 2014). Unsurprisingly, the antigen processing and presentation pathway has been shaped during the past 6,000 years. The introduction of farming, which led to exposure to a huge variety of new pathogens, as well as other civilization factors such as urbanization (thus increasing population density, insufficient sanitation, peridomestic animals, etc.) and development of trading routes increasing the probability of disease spread, etc., has changed the pathogenic environment drastically. Major pandemics, such as the plague in Europe, could have also played a very important role in the selection of immune system genes (Barnes et al. 2011; DeWitte 2014; Laayouni et al. 2014). It is quite possible that modern medicine has also been modifying the genetic mechanism of immune response. This issue still needs extensive research.

Graft-versus-host disease is considered by clinicians to be a disorder but from the evolutionary point of view it represents a powerful system of immune response to alien agents. This alloimmunity is evolutionarily ancient and seems to be an "unavoidable consequence" of a natural mechanism of antigen processing and presentation (Lakkis and Lechler 2013). Indeed, graft-versus-host disease shares 71% of common genes with the antigen processing and presentation pathway (most of them are *HLA*-genes) (supplementary table 7, Supplementary Material online). Being an inseparable part of the human defense system, alloimmunity should evolve together with it. Therefore, we suppose that all the abovementioned factors that caused changes in the antigen processing and presentation process should act similarly on the graft-versus-host disease pathway.

Another pathway connected with antigen processing and presentation is connected to autoimmunity. We revealed selection signals for autoimmune thyroid disease. It shares 37% of genes (all of them belong to *HLA* group) with the antigen processing and presentation pathway (supplementary table 7, Supplementary Material online). Therefore, the emergence of this autoimmune disease is probably a cost of the fast adapting antigen processing and presentation system; however, we believe that there are additional environmental factors that contributed to the intensive evolution of this particular disorder. Autoimmune thyroid disease is a syndrome characterized by chronic inflammation of the thyroid. It is believed to be specific for *Homo sapiens* (Aliesky et al. 2013) but it is unknown when this disease appeared in the human population. Currently, autoimmune thyroiditis is quite common in the European population (Vanderpump 2011). It can probably be connected with the increased carbohydrate uptake after introduction of agriculture which, in turn, has increased thyroid hormone levels in the human body (Kopp 2004). Increased levels of thyroid hormone, especially in combination with inappropriate iodine supply, cause several detrimental systemic disorders (Motomura and Brent 1998; Kopp 2004). Therefore, we assume that the emergence of new nonsynonymous mutations is probably an organismal reaction to this new hormonal status. Hypothetically, this reaction could be a kind of prevention mechanism, or, on the contrary, a consequence of thyroid hyperfunction.

We revealed significant changes in the PI3K-Akt signaling pathway. It is one of the universal signaling pathways, which are active in most of the human body's cells. It is responsible for a variety of fundamental processes, such as apoptosis, cellular growth, proliferation, cell survival, metabolism, and others (Song et al. 2005; Engelman et al. 2006; Duronio 2008; De Santis et al. 2017). This pathway was shown to play an important role in immunity, cancer, and long-term potentiation (Fresno Vara et al. 2004; Hou and Klann 2004; Sui et al. 2008; Weichhart and Saemann 2008; Porta et al. 2014; Chen et al. 2017; Pons-Tostivint et al. 2017). The PI3K-Akt signaling pathway is activated by different stimuli including antigens, inflammation, environmental toxicants, and drugs (Song et al. 2005; Engelman et al. 2006; Duronio 2008; De Santis et al. 2017). Therefore, any of the factors described above (changes in diet, pathogen environment, xenobiotics) could affect this pathway and stimulate accumulation of nonsynonymous mutations in it.

The hypertrophic cardiomyopathy (HCM) pathway also shows signals of selection during the past 6,000 years. HCM is an autosomal dominant disease, which is manifested as a functional impairment of the heart. It occurs in approximately 0.2% of modern populations (Cirino and Ho 1993; Marian 2010). The course of the disease is very often asymptomatic; however, in some cases, especially with intensive physical activity, a sudden cardiac death can occur. For example, hypertrophic cardiomyopathy is the leading cause of sudden cardiac death in young athletes (American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines et al. 2011; Barsheshet et al. 2011). We suppose that the higher prevalence of nonsynonymous SNPs in the modern group in comparison to the ancient group can be a consequence of the gradual change in European lifestyle from pretechnological agrarians to modern postindustrial societies: a redistribution of physical load, as well as of balance between calorie uptake and physical activity (Lightfoot 2013). Genetic monitoring and adequate therapy (American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines et al. 2011; Cirino and Ho 1993) probably also play a role in the accumulation of HCM-associated mutations in modern Europeans and, therefore, one can expect an even higher frequency of these mutations in the future.

Olfactory transduction, the capacity to discriminate odors, shows the strongest signal of selection (table 1). As reported before, olfactory genes in primates have a tendency to pseudogenization (Gilad, Man, et al. 2003; Pierron et al. 2013; Somel et al. 2013). In humans, approximately 60–70% of olfactory genes are pseudogenes; this probably reflects a decreasing need for olfactory perception in great apes and especially in humans (Rouquier et al. 1998; Gilad, Bustamante, et al. 2003). Indeed, relaxed selection has been described for most human olfactory genes (Gilad, Bustamante, et al. 2003; Somel et al. 2013) leading to fast accumulation of mutations in these genes (Miyata and Hayashida 1981; Gilad, Man, et al. 2003). According to our results, this process has also been taking place during recent human microevolution. Most likely, the process of pseudogenization of olfactory genes is still ongoing. At the same time, we cannot exclude the possibility that introduction of new cultural practices (new types of food, perfume, etc.) provides new directions for selection, at least for some olfactory genes.

All the pathways described above have been accumulating nonsynonymous mutations during the past 6,000 years. At the same time, we revealed three pathways with the opposite pattern: The modern group has significantly fewer mutations than the ancient one. These pathways are described below.

We revealed significant changes in a pathway associated with oocyte meiosis. Oogenesis is the most important part of female reproductive function. It determines the timing of puberty and menopause as well as the effectiveness of reproduction. It has been shown that all these parameters are strongly influenced by environmental factors (Gluckman and Hanson 2006b; Gold 2011; Henneberg and Saniotis

2013). Retrospective analysis and direct data suggest significant fluctuations in menarche onset during the past several millennia (Gluckman and Hanson 2006a, b; Gillette and Folinsbee 2012; Henneberg and Saniotis 2013); a tendency has been reported for later menopause in modern European women, which is probably connected with lifestyle and overall quality of life (Gold 2011). Several hypotheses discuss the fluctuations in the number of childbirths: Alterations in nursing and dietary habits in early agriculturalists might have caused a shortening of birth intervals (Kolata 1974; Hewlett and Lamb 2005; Gluckman and Hanson 2006a, b; Gold 2011), and subsequent introduction of artificial childbirth reduction (including induced abortions and, later, contraception) decreased the number of pregnancies. In turn, all these changes affected the number of menstrual cycles during a woman's life. Overall, it is expected that changes in the duration of the reproductive period and in the number of maturing oocytes might affect oocyte meiosis. The decrease in the number of nonsynonymous mutations in the oocyte meiosis pathway during the past six millennia probably implies that despite all environmental changes, in Europeans there was a tendency to keep the organism's homeostasis in such an important process as reproduction. The other possibility can be a shift in the mutation spectrum in order to adapt to the new environmental conditions.

Two more pathways (long-term potentiation and dopaminergic synapse) for which the number of nonsynonymous substitutions in the modern group is significantly less than in the ancient group are associated with cognitive functions, especially memory and learning. Information is probably the most rapidly changing factor of our environment. During the past millennia, ways of information presentation and perception have been completely altered. Six thousand years ago information was being accumulated from a relatively small geographic area and changed relatively slowly. With the evolution of transport and transmission techniques information capacity has expanded globally, and the quantity and quality of data to process have been markedly enlarged. Moreover, the main cognitive tasks in Europe have also dramatically changed during this time period (e.g., tool-making vs. car driving). This presumably affects such information perception systems as learning capability and memory. However, mutations in cognitive function genes can lead to detrimental consequences (indeed, mutations in genes in long-term potentiation and dopaminergic synapse pathways can cause schizophrenia, obsessive-compulsive disorder, Parkinson's disease, drug addiction, and many other neurological and neuropsychiatric disorders; Bibb 2005; Centonze et al. 2005; Kauer and Malenka 2007). These deleterious mutations should be eliminated through strong selection, both directly and indirectly via sexual selection connected with behavioral reactions. Data on molecular evolution of the human brain are still controversial, but most researchers suggest that coding regions of most human brain genes are subjects of negative selection (Miyata et al. 1994; Duret and Mouchiroud 2000; Hill and Walsh 2005; Tuller et al. 2008; Huang et al. 2013). Our results agree with this suggestion; at the same time, the observed trend can indicate directional changes as a response to the modified cognitive tasks.

In summary, we have revealed selection signatures in functional processes responsible for metabolic transformations, immune responses including protection against pathogens, alloimmune and autoimmune reactions, signal transduction, physical activity, sensory perception, reproduction, and cognitive functions. Interestingly, different environmental factors have induced different types of natural selection. An increase in the number of nonsynonymous mutations in modern humans can indicate signs of either positive or relaxed selection, whereas a decrease suggests negative or, on the contrary, strong positive selection. For the identification of the exact type of selection an additional analysis is required.

The weakness of our approach is that it is impossible to identify selection signals caused by modifications in a single gene (like, e.g., it was done in the work of Mathieson et al., 2015). Instead, it is possible to reveal multiple modifications in pathways that are the result of many weak signals undetectable by using other methods. Therefore, we believe that our results complement existing data on recent selection in the European population. Based on our results, we suppose that the most important civilization events that have affected adaptive reactions are changes in diet and the pathogenic environment, the introduction of xenobiotics, modifications in lifestyle and in the information background. To our knowledge, our work is the first evidence for natural selection on the functional level. Our results show that even during a relatively short period of time, the human genome can be significantly shaped by selection if the selection is induced by man.

Our results raise a number of questions, namely, when did selection began to influence the revealed processes? How their subsequent evolution was affected? To address these issues, further analyses on previous (Early/Middle Neolithic, Paleolithic) and intermediate (Iron Age, Middle Ages) time periods should be performed. We are convinced that, with the emergence of new data, we will better understand how deeply and how rapidly biochemical and metabolic pathways can be affected by cultural and social changes.

Materials and Methods

Ancient Data Preparation

We used published data from 159 European samples dated 3,500–1,000 BCE (Gamba et al. 2014; Allentoft et al. 2015; Haak et al. 2015; Mathieson et al. 2015). The focus of our investigation was the Bronze Age; however, since the borders between different archeological cultures and time periods are blurred, we also used samples attributed to the Late Neolithic (supplementary table 1, Supplementary Material online). Selected individuals probably spoke Indo-European family languages that currently prevail in Europe (Haak et al. 2015). Most of the Late Neolithic/Bronze Age individuals have been previously shown to be genetically related to Yamnaya culture (Lazaridis et al. 2014; Allentoft et al. 2015; Haak et al. 2015) and to most modern European ethnic groups (Allentoft et al. 2015; Haak et al. 2015).

According to the authors (Allentoft et al. 2015; Haak et al. 2015), genomic reads successfully passed quality controls on mitochondrial and bacterial DNA contamination. To ensure authenticity and remove batch effects, we used a Bayesian approach implemented in mapDamage 2.0 (Jonsson et al. 2013). We trimmed the past two nucleotides from each sequence; we further restricted our analyses to sites with base quality $\geq$20. To achieve statistical significance of the results we implemented the pipeline described in figure 2 and briefly outlined below. SNPs were called independently in every sample, filtered by mapping quality ($Q > 30$) and SNP quality (QUAL $>$20); if possible alleles were supported by the same number of sequence reads, we selected an allele at random. We set the allele to "no call" if the position was not covered by sequence reads. Genotypes for samples were called using the "call" command of *bcftools* (samtools, bcftools) (Li et al. 2009) and filtered for quality score (QUAL $\geq$20) and the coverage was required to be at least three per sample.

We calculated the density and number of nonsynonymous SNPs per sample (supplementary table 6, Supplementary Material online). We excluded eleven samples from analysis based on 1) absence of genotypes in every position, 2) outgrouping during PCA analysis shown in the original publication (supplementary table 2, Supplementary Material online), or 3) due to enormous SNPs numbers compared with other samples. For this, we computed the proportions of SNPs in the samples through all the 305 pathways in the KEGG database. To filter the samples, we calculated the proportion of SNPs in every sample for every pathway and then acquired the kernel density distribution for median proportions of SNPs per sample per pathway. The samples which were outside the 99th percentile were rejected from further analysis. The 99th percentile for the median proportion of SNPs per pathway was 7.0%, whereas the samples RISE98, RISE00, and RISE423 had average relative proportions of SNPs per pathway of 7.1%, 7.0%, and 15.1%, respectively. Therefore, these samples were rejected from further analysis. The final Bronze Age subset consisted of 150 samples (supplementary table 2, Supplementary Material online).

The resulting SNPs were annotated with the ANNOVAR (Wang et al. 2010) tool using the *hg19* human genome annotation and the *refGene* database (http://varianttools.sourceforge.net/Annotation/RefGene). Synonymous and nonsynonymous SNPs were pooled into 2 separate single data sets, resulting in a collection of 40,573 synonymous SNPs and 48,860 nonsynonymous SNPs, respectively. Next, we calculated the numbers of synonymous and nonsynonymous SNPs per KEGG pathway.

Modern Data Preparation

Modern data were obtained from the latest release of the "1000 Genomes Project" database (Genomes Project) (http://www.internationalgenome.org). We selected data only for European populations with Indo-European roots. Originally, the European subset includes British, Finnish, Spanish, Italians, and Utah residents with Northern and Western European ancestry. First, we excluded the Utah residents: Although they have European ancestry, the past several centuries they have been living in different geographical and cultural conditions, having different lifestyle, different diet, etc. (Willett et al. 2006). Next, we excluded Finnish, since their population history is different from other European populations (Lao et al. 2008). The Modern data set contained 305 individuals: 91 from the British population in England and Scotland, 107 from the Iberian peninsula (Spain) and 107 individuals from Toscani (Italy). SNPs were functionally annotated with the ANNOVAR tool (Wang et al. 2010) using the hg19 human genome annotation and the *refGene* database.

Depth Files Correction

Due to poor data sequence coverage, even after aggregation of sequence reads from all the Bronze Age samples, complete genome coverage had not been achieved. Prior to calculating the distribution of nonsynonymous SNPs in the Bronze Age and modern Europeans, to avoid artificially high enrichment scores, we restricted our analysis of modern Europeans to genomic positions covered by the Bronze Age sequence reads. SAMtools (Li et al. 2009) was used for coverage calculation, then the results were filtered to keep coverage above 3 and mapping quality above 30 ($Q > 30$). We generated a list of covered bases and used this list to select those SNPs in the modern human subsets that are covered in the Bronze Age samples. After this filtering, the modern subsets contained 72,558 synonymous and 96,710 nonsynonymous SNPs.

KEGG Annotation and Preparation for Enrichment Analysis

Distribution of SNPs in Genes

A combined lists of 1) synonymous SNPs and 2) nonsynonymous SNPs from the Bronze Age individuals and present-day Europeans was mapped onto 305 KEGG pathways (Du et al. 2016), and counts of SNPs per pathway were computed. To minimize the false-positive rate, we included only pathways containing more than five genes with SNPs and with sum covered pathway length more or equal to 50% in aggregated ancient data (table 1 and supplementary table 2, Supplementary Material online).

Enrichment Analysis

To analyze differences in numbers of SNPs per pathway between the Bronze Age and present-day individuals, we calculated 1) DSSE scores and 2) DNSE scores. The calculations for DSSE and DNSE were performed in a same way; below, we describe the calculations for DNSE.

First, we calculated the number of nonsynonymous SNPs in both the ancient and modern groups. We assume that during neutral evolution similar pathways accumulate nonsynonymous SNPs at the same rate, and during enrichment analysis such pathways would fit a normal distribution, whereas pathways that are affected by evolutionary pressure would be outliers from this distribution (supplementary fig. 4, Supplementary Material online). If $K = 305$ is the total number of studied pathways, and $i = 1,\ldots, K$, number of the pathways, in Bronze Age and modern samples, n_i and m_i denote the number of nonsynonymous SNPs per ith pathway. The

expected (equilibrium) fraction of nonsynonymous SNPs in ancient data is given by $p = n/(n + m)$, where p is the fraction of ancient nonsynonymous SNPs in the whole analyzed subset, n is the amount of ancient nonsynonymous SNPs in KEGG pathways, m is the amount of modern nonsynonymous SNPs in KEGG pathways. The fraction p_i of ancient nonsynonymous SNPs in the ith KEGG pathway is $p_i = n_i/(n_i + m_i)$, where n_i is the amount of ancient nonsynonymous SNPs in the ith pathway, m_i is amount of modern nonsynonymous SNPs in the ith pathway. From acquired numbers enrichment DNSE scores were computed for every pathway with continuity correction (Fleiss et al. 2003):

$$\mathrm{DNSEScore} = \frac{(p - p_i) \pm \frac{1}{2(m_i+n_i)}}{\sqrt{\frac{p(1-p)}{m_i+n_i}}}.$$

After computing the DNSE scores (distributed normally, Shapiro–Wilk test P-value >0.01), we calculated P-values using Bonferroni and Benjamini–Hochberg corrections and identified the differentially enriched pathways. The pathways were considered to be differentially enriched if absolute value of the DNSE score >4, and the adjusted P-value <0.01. However, in 2017 in Nature Human Behavior (Benjamin et al. 2017) the manuscript "Redefine statistical significance" was published, where it was proposed to decrease the P-value threshold from 0.01 to 0.005. We implemented the proposed threshold on our data to avoid further false-positive enrichment signals resulting in alternative lists of enriched pathways.

In order to normalize nonsynonymous SNPs on synonymous SNPs, we performed the following procedure. Bonferroni-adjusted P-values were log-transformed (base 10) and multiplied by the sign of the DNSE statistic, so that positive scores correspond to enrichment in modern groups and negative scores to enrichment in ancient groups, respectively. As a condition of significance, we required the following: The P-value of the nonsynonymous test was below the P-value of the synonymous test for each pathway. In addition, it was required for the Bonferroni-corrected P-value to be below 0.01. For each pathway, the P-value of the synonymous test was above the P-value of the corresponding nonsynonymous test.

Validation of the Method

To validate our method, we compared it with the method implemented by Somel et al., 2013. We calculated DNSE scores between chimpanzee and their ancestors (combined genomes from different species of primates; data from Prado-Martinez et al., 2013, https://www.nature.com/articles/nature12228). Our results confirmed the conclusions of Somel et al.: Olfactory transduction pathway demonstrated the signature of relaxed selection in chimpanzee (enriched in comparison with primates; it is the only pathway enriched in chimpanzee) (supplementary table 8, Supplementary Material online). As in Somel et al., 2013, proteasome pathway did not demonstrate any signs of selection (no pathway enrichment) (see Somel et al., fig. 2 and present study, supplementary table 8, Supplementary Material online). We also revealed several pathways which are enriched in primates in comparison to chimpanzee. This can indicate possible negative or strong positive selection in chimpanzee. However, this suggestion requires additional thorough analysis which is outside the scope of our paper.

Comparison of Regulatory SNPs Distribution

To compare regulatory SNPs in Bronze Age and modern individuals, we extracted 10,000 experimentally validated promoters and 5′-UTRs from the DBTSS database (https://dbtss.hgc.jp). Sequences [TSS −1,000, TSS + 1,000] were extracted and MATCH software with TRASNFAC database (https://www.ncbi.nlm.nih.gov/pubmed/12824369, with parameters set to minimize false-positive matches) was applied to identify putative transcription factor binding sites (TFBS) in those sequences. A total of 61,451,840 putative TFBS were identified in these regions. The TRANSFAC database is highly degenerate with different entries having the same or similar matrices, therefore producing overlapping predictions on a genome. Such overlapping putative TBFS were merged into 88,513 contiguous regulatory sequences. Furthermore, we removed those regions that were not fully covered by ancient DNA sequences, leaving us with 31,036 regulatory fragments. A proportion test was performed similarly to the calculation of enrichment scores in coding regions.

PCA and reAdmix

The principal component analysis (PCA) was carried out in R using the ADMIXTURE vectors for Ancient and European/Worldwide modern individuals. The ADMIXTURE software implements a model-based Bayesian approach that uses a block-relaxation algorithm to compute a matrix of ancestral population fractions in each individual (Q files) and infer allele frequencies for each ancestral population (P files) (Alexander et al. 2009; Alexander and Lange 2011). We applied ADMIXTURE in *unsupervised* mode to the combined data set of modern and ancient individuals. We varied the number of components between $K = 6$ and $K = 17$, recording the value of cross-validation (CV) error and picked $K = 7$ for the PCA analysis as a sufficient number of components to distinguish subpopulations from each other.

The PCA analysis was performed using the R package princomp with centering and scaling parameters and then visualized using the first two components cumulatively corresponding to 60% of the variance among worldwide modern and ancient individuals.

Additional ancient samples for reAdmix (Kozlov et al. 2015) analyses were obtained from (Gamba et al. 2014; Allentoft et al. 2015; Haak et al. 2015; Mathieson et al. 2015). This data set was combined with the modern European samples from the 1,000 Genomes database. The resulting data set contained 1) the Bronze age subset used in this study ($n = 150$), 2) early Neolithic data ($n = 32$), and 3) Western European hunter-gatherers ($n = 12$). A reference data set was assembled from all ancient individuals, and modern individuals were represented as a linear combination of

ancient ones using the reAdmix algorithm. Each modern population was represented as

$$\text{Modern Population} = w_1\text{BA} + w_2\text{EN} + w_3\text{WHG} + \varepsilon,$$

where BA is "Bronze Age", EN is "Early Neolithic", WHG is "Western Europe hunter-gatherers" data, ε is an unassigned part, and coefficients were determined using the differential evolution algorithm. Modern individuals from 1,000 genomes (British population [$n = 6$], Toscani [$n = 6$], and Iberian [$n = 5$]) were clustered within self-reported ethnic groups based on similarity of their admixture vectors, and the self-reported identity was validated using leave-one out procedure and Euclidian distance to the reference population. Average contributions of ancient genomes to modern individuals were computed for each cluster of modern individuals.

Data Access

SFTP access with ANNOVAR annotated vcf files for ancient man and filtered nonsynonymous and synonymous files from modern samples:

ip 85.89.112.202
port 2203
username: bronze_man
password: bronze_man

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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