

## Research Article

# Of Wheat and Men: Changes in Genetic Markers for Celiac Disease Over Time

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## ABSTRACT

In the recent past, sequencing of ancient human genomes has become increasingly common, leading to an immense amount of data to be explored. For this study we focused on comparing a set of ancient individuals with modern populations on behalf of markers for celiac disease. We analyzed a panel of 64 SNPs related to this disease, trying to detect changes in allele frequencies between ancient and modern individuals. We hope to make a contribution to the subject of genetic health throughout human history.

## KEYWORDS

Ancient DNA; Celiac Disease; Allele Frequencies; Risk Factor

## INTRODUCTION

### The sprout to pre-eminence

Cereals, particularly wheat, are a mainstay of traditional western diets. Modern wheat kernels consist to 70 to 75 percent of proteins, divided into glutenin and gliadin which can form several polymers summarized under the term "gluten" [1]. They all share a unique amino acid composition with a high content of glutamine and proline and only low contents of amino acids with charged side groups [2]. In the wheat plant, gliadin and glutenin serve primarily as storage for nutrients [3]. The gluten polymer serves as "glue" in dough, providing stability and elasticity. Therefore wheat is a popular ingredient in bread, cakes and other bakery goods, beer and many more of the foods an adult might consume throughout the day. The last 50 years have seen a rate of increase in wheat consumption that is higher than for any other cereal. During this period in the United Kingdom, an average of 50% of the total carbohydrates consumed by individuals derived from cereals [4]. But the roots of wheat as a major source for carbohydrates stretch back much farther, to about 12500 years (BP), and the beginning of the Neolithic period [5].

### Historical roots

One of the oldest evidences of controlled planting of crops is dated around 21000 BP [6], but in general

wheat is believed to have played only a minor role in human diets during these times [7]. Until then, a nomadic lifestyle prevailed, sustained by hunting and gathering. Unprocessed, the ancient wheat was only an inferior source of carbohydrates and large scale farming was impossible, due to limiting climatic factors [7, 8]. During the span around 12500 BP the climate changed and this allowed the establishment of larger permanent settlements and an increased exploitation of grasses. The first archaeological evidence for successful cultivation of grasses in larger scales was found in the northern area of the Arabian Peninsula [7], the so called "fertile crescent" [9], and marks the onset of the Neolithic period.

### The "necereal" evil

Numerous studies have linked wheat consumption to an increase in the incidence of celiac disease, which is caused by an autoimmune reaction to gluten and other wheat proteins. Symptoms range from diarrhoea, abdominal pain, impaired growth, iron deficiency, anaemia and a decrease in bone density [10–12]. The predisposition to celiac disease, its symptoms and strength depend on lifestyle, environmental, and genetic factors. A number of genetic markers have been associated with celiac disease and its symptoms [13–15]. The SNPs with the strongest association towards celiac disease are found within a region called the HLA-loci located on chromosome 6. Two of these, HLA-DQ8 and HLA-DQ2.5, are routinely used in clinical diagnoses of celiac disease [16]. These variants usually have a weak penetrance and are found in up to 40% of the unaffected population as well [17]. Yet inheriting these specific mutations substantially increases a person's risk of developing celiac disease. Due to its incomplete penetrance, the gold standard for celiac disease diagnosis lies in tissue biopsies and IgA anti-tissue transglutaminase antibody tests [18].

The aim of the present study is to probe changes in allele frequencies in any of the SNPs associated with celiac disease. Changes and variations in the frequency of markers associated with celiac disease, during or after the domestication of plants, can potentially be linked to the increased wheat consumption and might have had an effect on the prevalence of the disease. To gather evidence on such potential allele frequency changes over time, we compared genomes of

ancient hunter-gatherers and farmers as well as modern individuals.

## MATERIAL AND METHODS

### Data

The ancient dataset was formed of 57 ancient humans, consisting of 15 Hunter-Gatherers and 42 Farmers. We used the data of ancient samples from different publications (Supplement S1), focusing on samples with available whole-genome-shotgun sequencing data.

All data for modern individuals was downloaded in VCF-format from the 1000 Genomes Project (1000 Genomes Project Consortium, 2010).

The selected SNPs outside the HLA-regions were chosen by a review of literature (Supplement S2).

### Data processing

The usage of data aligned to different reference genomes might lead to unreliable results. Therefore, BAM files, that were not aligned against the human reference hs37d5 (hs37d5.fa.gz file from [ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2\\_reference\\_assembly\\_sequence/](ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequence/)), were realigned. In brief, the BAM-data was reverted to fastq-format.

Then the alignment program bwa (v0.7.15) [19] in the bwa aln mode was used with standard options to map the data against the reference. The resultant BAM files underwent final processing including an indel-realignment (GATK toolkit v3.6) [20–22] and a quality-filter step (samtools v1.3.1) [23]. After every processing step, we validated the resulting BAM-files for the SAM-format specification using picard tools (v2.8.0) (<http://broadinstitute.github.io/picard>).

SNP-calling for all BAM files was performed using the AntCaller (v1.1) [24] with standard options, resulting in VCF files for all of the ancient data. Since the AntCaller does not create VCF header lines automatically, we had to add the lines needed for further data processing. All SNP-calling methods used for ancient data have a tendency to overestimate heterozygosity [25]; therefore, the files were filtered for a read depth over 2 and a genotype quality value over 30, using (bcftools, v1.4).

### Bioinformatics Methods

The analysis of the SNPs strongest correlated towards celiac disease inside the HLA-regions was conducted using the tool HLA-VBSeq [26]. We tested the functionality of the tool for ancient DNA successful, but due to time constraints, we focused our analysis on 64 SNPs outside the HLA-regions (Supplement S2).

All SNPs connected with celiac disease outside the HLA-regions were analysed within the provided VCF files. After merging these filtered VCF-files of the ancient data with the files for the modern reference individuals,

calculations for allele counts and allele frequencies were conducted using the program vcftools (v0.1.13).

### Statistics

In order to gauge the genetic risk for celiac disease per ancient individual, we implemented a simple risk score. The score for an ancient individual is calculated by first dividing all alleles present (number of existing genotypes x 2) by all alleles that can be possibly covered (number of SNPs x 2). Then this factor is taken times the sum for the risk alleles over all SNPs ( $n = 64$ ), whereby a homozygote occurrence of a risk allele counts as 2 and a heterozygote occurrence counts as 1. This formula uses no log odds ratio for disease risk for SNPs and assumes independence between SNPs. We choose this method, as we did not have access to samples that had a confirmed diagnosis of celiac disease in our modern reference data set.

$$\text{simple risk score} = \frac{\text{covered genotypes} * 2}{128} * \sum 2 * \text{homozygote for risk allele} + 1 * \text{heterozygote for risk allele}$$

For a comparison of risk factors between ancient and modern individuals, we used a method similar to Berens et al. 2017. For every ancient genome, we subsampled all the individuals in the 1000 Genomes Project to match the coverage for that respective ancient genome. Then, the simple risk score was calculated for every subsampled modern genome. The percentile of the ancient risk score, relative to the modern individuals was generated via the ecdf function in R.

In order to create a comparable dataset of modern individuals, we bootstrapped the superpopulations of the 1000 Genomes Project. In brief, we randomly selected 20 individuals from the respective population pool, calculated allele frequency and repeated this procedure 1000 times. The mean allele frequency and the mean allele count of the complete modern reference, as well as for every subpopulation (Supplement S3), were used with the ancient population via Fisher's exact test [27].

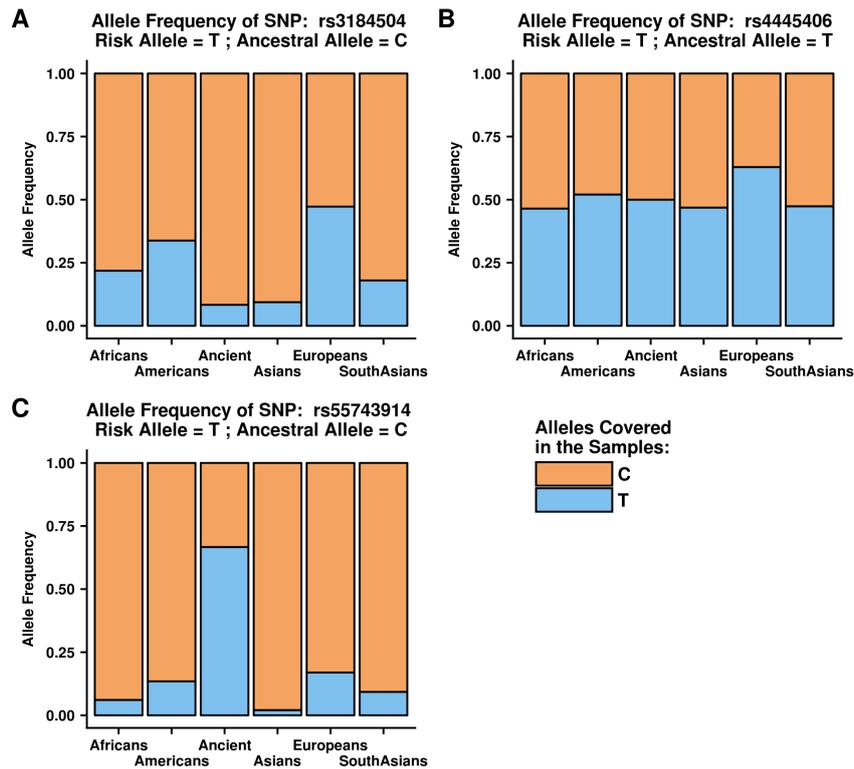
To get an overview of the genetic distance between our ancient samples and a modern European reference we used LASER (v2.04) to create a primary component analysis (PCA). Since all ancient samples originate from Eurasia, we chose a reference dataset [28, 29], which provides a better resolution for Europe.

All of the plots in this study were created using R (v3.4.2).

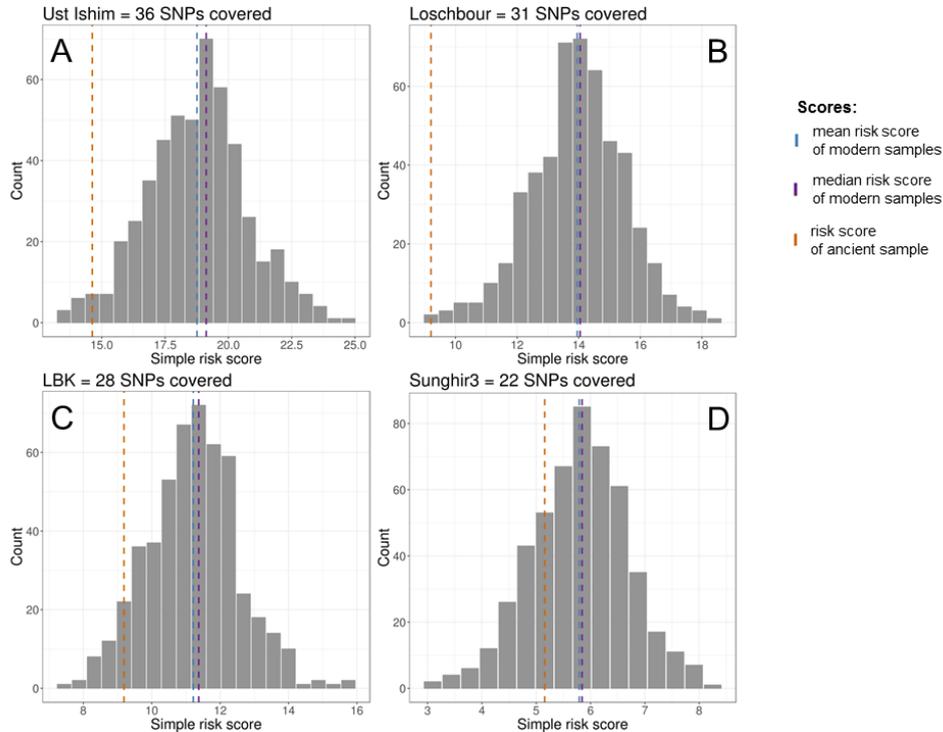
To maximize time efficiency, we used GNU-parallel whenever possible [30].

## RESULTS

From the 57 ancient individuals in our dataset, only 11 had at least one SNP covered. Out of the 64 selected loci outside of the HLA regions, our ancient samples contained 51 SNPs with a sufficient coverage and genotype quality (Supplement S2).



**Figure 1: Allele frequencies of three selected SNPs associated with celiac disease.** Risk variant in blue, non-risk variant in orange. Shown are 5 modern populations plus the ancient population.



**Figure 2: Subsampled risk scores for the respective ancient individual.** The risk score for the ancient individual is given in orange, mean and median for the risk scores of the modern individuals are given in blue and violet. The overall distribution of the respective risk score for matched modern Europeans is represented by the grey histogram in the background. Ust Ishim: Ancient sample from Ust’-Ishim in Siberia , Loschbour: Ancient sample from Loschbour in Luxembourg, LBK (Linearbandkeramik): Ancient sample from Stuttgart in Germany, Sunghir3: Ancient sample from Sunghir in Russia.

A general overview over the genetic distance between our samples and a modern reference is provided by a PCA (Figure 1, Supplement S5). It allows a rough estimate of which modern populations are closest to our ancient samples while showing at the same time the distance in between the ancient dataset.

For an overview over our results, we selected 3 representative SNPs with sufficient coverage that showed different trends in allele frequency.

The SNP rs3184504, located in the gene SH2B3, shows a shift towards an increase in the frequency of the risk allele in the modern European populations (p-value = 0,01811) (Figure 1A & Supplement S3).

In contrast, the SNP rs4445406, an intron variant of the gene MMEL1, shows no significant fluctuations of its frequencies between the ancient and the modern European population (p-value = 0,46268) (Figure 1B). A decrease of the risk allele frequency is detectable between ancient and modern European individuals for the SNP rs55743914 (p-value = 0,04502) associated with the gene PTPRK (Figure 1C).

Inspired by [31], who used a de-novo assembly approach to identify specific immune loci, we tried a different method, using HLA-VBSeq. This tool is a genotyper specific for HLA-regions which are highly diverse and therefore not suitable for normal alignment. We took the raw fastq data of the sample from Satsurbliia Cave (SATP), of which we extracted the unmapped reads, using an aligned BAM file as reference. We successfully applied this approach to this ancient sample and found it is suitable for ancient DNA in general. We were unable to detect HLA-loci associated with celiac disease.

Eleven ancient individuals exhibited a coverage at least 1 SNP on the selected 64 positions (Supplement S2). However, most individuals had a negligible coverage of at most 3 SNPs. Only 4 individuals exhibited a coverage of more than 20 SNPs. For all of the better covered individuals, we calculated a simple genetic risk score. This risk score reflects a presence/absence of risk alleles for celiac disease in the respective ancient individual. For each ancient sample, we additionally subsampled modern Europeans from the 1000 Genomes Project to the same SNP coverage and calculated a risk score distribution for matched modern healthy genomes in order to be able to compare them to our ancient samples (Figure 2 and Supplement S4).

In all 4 cases the risk score of the ancient individuals was below the mean of the risk scores of the modern healthy Europeans from the 1000 Genomes Project.

## DISCUSSION

The results of our data analysis provide a good overview of a wide spectrum of changes in allele frequencies for 64 SNPs connected to celiac disease (Figure 1) and their combined risk score for representative individuals (Figure 2). We cannot make any significant claims towards a general selection

at any of the observed sites, since our ancient dataset does not provide the necessary coverage for those kinds of analyses. Nevertheless, there are certain trends observable in several SNPs.

In the case of celiac disease, an increase of the frequency in favor of the risk allele might be explained with an evolutionary advantage surpassing the negative effects of the increased risk to suffer from the disease. In cultures which are mostly sustained through agriculture, an increase of the risk for celiac disease could mean a painful disadvantage. However, certain factors might still lead to an increase of the allele frequency for the risk allele. Aside from genetic drift, a random change in allele frequencies, the risk allele might be related to another phenotype beneficial for the organism.

The risk variant of the SNP rs3184504, for example, is thought to be connected with the immune system, and to possibly enhance the prevention of bacterial infection [32] due to its presence in the gene SH2B3. This hypothesis is supported by our data, showing an increased allele frequency for the risk allele in the modern populations, especially in the European population, compared to the ancient individuals (Figure 1A). These changes in this specific allele are generally associated with a selective sweep which occurred between 1200 and 1700 years ago, possibly triggered by an infectious disease.

Aside from a shift towards an increased risk allele frequency, there is the opposite possibility: Changes against the risk allele. A decreasing risk allele frequency can be caused, apart from the ever present genetic drift, by a negative influence on the health of the individual. An example for decreasing allele frequency presents itself in form of the SNP rs55743914, located in the gene body of PTPRK. Our data shows a significant difference between the ancient population and the modern ones, with a decrease in risk allele frequency on the modern side (Figure 1B). This SNP is associated with celiac disease [33], which could be a possible factor for the shift.

After crossing out increases and decreases in the risk allele frequency there is the third possibility: no change whatsoever in the allele frequency. No significant changes in the allele frequency, besides the normal fluctuations caused by the genetic drift, might indicate an absence of any advantages or disadvantages for the affected individual. An example from our data can be seen in the SNP rs4445406, associated with the gene MMEL1 (Figure 1C). There is no known connection towards phenotypes besides celiac disease, and the small fluctuations in allele frequency might signify no strong impact of the SNP on the prevalence of the disease.

In case of the overall simple risk factor for celiac disease, the individuals with coverage greater than 20 SNPs were generally below the majority of matched healthy genomes from the European population of the 1000 Genomes Project, with regard to their risk factor. Given the fact of lacking coverage, especially for the loci that are most associated to the disease, this is a trend at

best. Even if the coverage were sufficient, it is worth to mention that all identified SNPs that increase a risk for a specific disease, were found by comparisons of modern individuals and might not hold true for comparison between ancient and modern individuals. Additionally, a heightened genetic risk does not necessarily mean that an individual will develop the disease. To date it is unknown, as to whether “genetic health” has increased or decreased over the last few thousand years and several conflicting theories exist [34, 35]. A recent study performed by Berens et al., 2017 tested over 100 ancient genomes for the presence or absence of a broad panel of disease-related SNPs. They seemed to find a trend for a decrease in genetic risk over time, albeit not significant. All in all, the genetic health of ancient individuals is certainly an interesting topic for future research.

While we did find coverage on some HLA-loci for SATP, in processing the data with HLA-VBSeq, there was no correction of the C to T shift. There are several ways to correct this shift, but completely eliminating it would have exceeded the scope of this study. Furthermore there is the possibility of misalignment and bias within the results. The fragmented state of ancient DNA may lead to spurious mapping, exacerbated by the fact that the mapping process involves a database of all known HLA-variants. None the less, we consider this to be an appropriate approach for further studies.

There are several factors in our study, which might lead to biased results. First and foremost, our sample size is very limited, which might lead to wrong signals, due to uncommon variations. Another problem presents itself in the form of demography. All our results could be influenced by founder effects and genetic bottlenecks. Additionally, it must be considered that the individuals from the ancient dataset are very widespread in terms of time, geography and ancestry. This, together with the incomplete penetrance of the disease and additional environmental factors, make it difficult to claim results as unbiased.

The next steps in research would be a repeated analysis with a bigger dataset, together with data from patients afflicted with celiac disease. Improved statistical methods and population analysis, like the calculation of FST-Values, might shed new light upon this research subject. Another important step is the inclusion of demography, which might be another influence on the distribution of the SNPs.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## SUPPLEMENTARY DATA

High resolution figure files and supplementary files are available at [Genomics and Computational Biology online](http://Genomics and Computational Biology online).

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