Distribution of the -13910C>T polymorphism in the general population of Portugal and in subjects with gastrointestinal complaints associated with milk consumption

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Running title: -13910C>T polymorphism in the Portuguese population

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Abstract

Background: The -13910C>T polymorphism has been associated with lactase persistence (LP) in European populations.

Aim: To assess -13910C>T genotypes across Portugal and in adult individuals with unspecific gastrointestinal complaints associated with milk consumption.

Subjects and methods: We genotyped -13910C>T in general population from Northern (n=64), Central (n=70) and Southern (n=65) Portugal, and in 40 subjects with gastrointestinal symptoms. Additionally, we evaluated the concordance between breath-hydrogen test and -13910C>T genotypes in 65 samples.

Results: An overall frequency of 0.349 for the LP -13910*T allele was estimated in the general population, with a noticeable decrease in the South (0.269) compared with North (0.383) and Centre (0.393). Among the symptomatic group, the frequency of the -13910*T allele (0.363) was not significantly different from the general population. We found a 94% concordance between the breath-hydrogen and the molecular tests.

Conclusions: Our study suggests that i) the distribution of the LP polymorphism is not uniform across the country, ii) genotyping -13910C>T is a good diagnostic tool for lactase status in the Portuguese population, and iii) self reported gastrointestinal complaints are not good predictors of the LP status, implying that a significant part of those complaints may not be related to hypolactasia.

Key words: Lactase persistence; lactose intolerance; gastrointestinal complaints; polymorphism -13910C>T; Portugal**.**

Introduction

Lactose intolerance is an autosomal recessive condition caused by the deficiency of the lactose-hydrolysing enzyme lactase (i.e., adult-type hypolactasia) and it is an important cause of gastrointestinal complaints. In most human populations the ability to digest the sugar lactose present in milk starts to decline after the weaning phase because of a decreased expression of the enzyme lactase in the small intestine (Swallow, 2003). In these lactase non-persistence (LNP) or lactase restriction cases, individuals are unable to digest significant amounts of lactose and suffer adverse unspecific symptoms after ingestion of dairy products, including abdominal pain, cramps, nausea, flatulence or diarrhoea (Haberkorn et al. 2011). Lactose intolerance is usually investigated by indirect laboratory methods including breath hydrogen test (BH_2T) (Newcomer et al. 1975) or plasma glucose levels (Arola, 1994) after lactose ingestion.

While LNP is the ancestral condition in humans, a substantial number of individuals with European ethnic ancestry maintain the ability to digest milk. This lactase persistence (LP) phenotype reaches its highest frequencies in north-western Europe (80%–95%), showing a decreasing cline to the south and east Europe (~50%) (Ingram et al. 2009; Itan et al. 2010). The persistence of the lactase enzyme in adulthood was recently associated with several polymorphisms located ~14 kb upstream the lactase (*LCT*) gene, in intron 13 of the adjacent *MCM6* gene (Ingram et al. 2009). The single nucleotide polymorphism (SNP) -13910C>T (rs4988235), was shown to be strongly associated with increased lactase activity in Northern European populations: genotype CC_{-13910} exhibited a complete association with hypolactasia, whereas genotypes CT and TT correlates with lactase persistence (Enattah et al. 2002). Genotyping -13910C>T has been subsequently tested as a diagnostic tool to determine lactose intolerance in European populations (Ridefelt and Hakansson 2005; Kerber et al. 2007; Krawczyk et al. 2008; Mattar et al. 2008; Pohl et al. 2010; Haberkorn et al. 2011).

Lactase persistence is also a common trait in many African, middle Eastern and southern Asian pastoralist groups but the persistence -13910*T allele was found in only a few West African pastoralist tribes (Mulcare et al. 2004; Lokki et al. 2011). Additionally, other variants located in the same enhancer region upstream of the *LCT* gene, including -13907C>G, -13915T>G and -14010G>C, have been shown to be associated with lactase

persistence in different parts of East Africa (Tishkoff et al. 2007; Enattah et al. 2008) and the Arabian peninsula (Imtiaz et al. 2007).

The purpose of this study was i) to assess -13910C>T genotypes in the general Portuguese population clustered into the three main regions of mainland Portugal (North, Centre and South), and ii) to test if self reported gastrointestinal complaints after milk ingestion are good predictors of the LP status. Furthermore, we also evaluate the concordance between the lactase status determined by breath-hydrogen test and by -13910C>T genotypes in Portuguese family samples.

Materials and Methods

Samples

General populations: A total of 199 non-related, randomly chosen, native individuals from Portugal North (above Douro river; n=64), Centre (between Douro and Tagus rivers; n=70) and South (below Tagus river; n=65), were analyzed for the -13910C>T polymorphism.

Subjects with gastrointestinal complaints: The symptomatic study group comprised 40 adult Portuguese subjects of European descent, mainly from Central and Northern Portugal (17 males and 23 females; age range: 19-58 years; mean: 32.7 years), recruited from the general population as volunteer donors, otherwise healthy but self reporting unspecific gastrointestinal complaints after milk consumption, including abdominal pain, bloating, flatulence, nausea and diarrhoea.

Concordance study: A concordance study between the molecular and the breath hydrogen tests was undertaken in 65 Portuguese pediatric cases referred for abdominal symptoms suggestive of lactase restriction and their first degree family members (age range: 5-68 years; mean: 18.4 years) recruited in Pediatrics Gastroenterology Unit of the University Hospital of Santa Maria (Lisbon).

All individuals included in this study, or their legal representatives, gave their written informed consent.

Molecular studies

Genomic DNA was extracted from buccal swabs using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden).

Genomic DNA was analyzed by a TaqMan allelic discrimination assay allowing the detection of the -13910C>T SNP alleles on a MiniOpticon™ Real Time PCR System instrument (BioRad Laboratories, Inc.). Primers, labelled probes and conditions were reported by Ridefelt and Hakansson (2005). Each run of 48 samples included three negative controls and one positive control of each genotype previously analyzed by sequencing.

Additionally, genomic DNA from subjects with gastrointestinal complaints was PCR-amplified using the forward primer 5'-TGCTCATACGACCATGGA-3' (designed), and the reverse LAC-C-L*2* 5'-CTGCTTTGGTTGAAGCGAAGAT-3' reported by Mulcare et al. (2004), between nucleotides -14116 and -13737 upstream *LCT* gene transcription initiation site, spanning the vicinity of LCT -13910C/T SNP, to screen for additional derived alleles that could influence the LP phenotype. The 379-bp PCR fragments were purified with ExoSAP-IT (Amersham Biosciences) and subsequently subjected to Sanger's dideoxy chain termination sequencing reaction using the Big-Dye Terminator v1.1 Cycle Sequencing kit (*Applied Biosystems, Foster City, USA*), and analyzed in an ABI 310 automatic sequencer *(Applied Biosystems, Foster City, USA).*

Breath hydrogen test (BH_2T)

The BH_2T was performed according to standard methodology: after a minimum of 6 hours fasting, 20% lactose aqueous was ingested, at a dosage of 2g/Kg body weight up to a maximum of 50g. Expired air samples were collected at minute 0 (previously to lactose ingestion) and at 30 minute intervals for 3 hours after ingestion of the lactose source. The hydrogen concentration in expired air was measured by gas chromatography on a *Lactoscreen* apparatus. Individuals with increments in hydrogen concentration of more than 20ppm above the baseline were diagnosed with LNP.

Statistical analysis

Allele frequencies were estimated by mere counting. Hardy-Weinberg equilibrium probability values, heterozygosity and exact p-values for population differentiations (Raymond and Rousset, 1995) were achieved using the software package Arlequin, v.3.11 (http://cmpg.unibe.ch/software/arlequin3/) (Excoffier and Schneider, 2005).

Results and Discussion

Genotype distributions and allele frequencies for the -13910C>T polymorphism across the study general Portuguese population are shown in Table I. We found an overall frequency of 0.349 for the lactase persistence -13910*T allele in accordance with previous reports in Northern Portugal (0.37) (Coelho et al. 2005), or Spain (0.386) (Agueda et al. 2010). However, we found that -13910*T allele frequencies in southern regions of Portugal (0.269) are lower than in the North (0.383) and Centre (0.393), and that these differences are very close to being statistically significant using a population differentiation test: p=0.05, North vs. South, and p= 0.06, Centre vs. South. This finding is in general agreement with the overall tendency for a North-South decreasing gradient of the -13910*T frequency in Europe (Ingram et al. 2009). In the general population, -13910*T allele frequency values are lower than those estimated in Northern European populations (average ~0.64) and Central Europe (average ~0.49) (Itan et al. 2010), and higher than those obtained in southern European populations from Greece (0.09) and Italy (ranging from 0.08 in South to 0.237 in North-East; mean frequency of 0.141) (Anagnostou et al. 2009).

Genotyping of 40 Portuguese adult subjects with unspecific abdominal complaints after milk ingestion showed that 16 (40.0%) have the CC_{-13910} homozygous genotype associated with lactase non-persistence; 19 (47.5%) were CT_{-13910} heterozygous and 5 (12.5%) were TT_{-13910} homozygous (Table I). Estimated allele frequencies were 0.637 and 0.363 for -13910*C and -13910*T, respectively (Table I).

Although we have not been able to perform a physiological lactose tolerance test in the symptomatic group, a comparison test between the results lactase status in 65 pediatric cases and relatives that were simultaneously typed with the molecular test and BH_2T was performed (Table II). We found a 94% (61/65) concordance between lactase status diagnoses

using the two tests. If the BH_2T is considered to be the standard, the false positive and false negative rates for genotyping are 10% (3/30) and 3% (1/35), respectively. Alternatively, if the molecular test is the standard, false positive and false negative rates for the BH_2T are 4% (1/28) and 8% (3/37), respectively. The latter values are similar to the error rates of the BH_2T calculated by Mulcare et al (2004) by combining the results of five different studies. The high correlation between the BH_2T and the molecular test observed in the present comparative study indicates that genotyping the -13910C>T polymorphism is a good diagnostic tool for lactase status in the Portuguese population. This is in line with different studies in European descent individuals also reporting excellent matches between lactase genotypes and LP/LNP assessed by the breath hydrogen test (Kerber et al. 2007; Krawczyk et al. 2008; Mattar et al. 2008; Pohl et al. 2010; Haberkorn et al. 2011) or plasma-glucose measurements (Ridefelt and Hakansson 2005).

About two thirds of the sampled Portuguese individuals (24/40; 60.0%) (Table I) presenting unspecific gastrointestinal milk problems, have the CT_{-13910} or TT_{-13910} genotypes associated with LP. Although the frequency of the persistence -13910*T allele in cases (0.363) was found to be slightly lower than in the controls randomly ascertained from Northern (38.3%) or Central (39.3%) Portugal, the difference is not statistically significant (exact p-values = 0.878, 0.640, respectively). This lack of significant differences between the symptomatic group and the general population suggests that many unspecific gastrointestinal complaints associated with milk ingestion - including abdominal pain, bloating, flatulence and diarrhoea - are not related to lactase non-persistence. Otherwise, a much higher frequency of the CC_{-13910} genotype should be expectable in the symptomatic group.

No other derived alleles were found by sequencing between nucleotides -14116 and -13737 upstream the *LCT* gene transcription initiation site in the Portuguese symptomatic group, including variants -14010G>C, -13915T>G and -13907C>G, previously associated with lactase persistence in some Afro-Arabian pastoralist populations.

In conclusion, our study suggests the existence of a population stratification in Portugal concerning the -13910C>T polymorphism. The study also shows that self reported gastrointestinal complaints associated with milk consumption in the Portuguese population

are not good predictors of the lactase persistence status and that a significant part of those complaints may not be related to hypolactasia.

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Table I - Genotypes and allele frequency distributions of -13910C>T polymorphism among the general population of Portugal and in subjects with unspecific gastrointestinal symptoms after milk ingestion.

Population	n	Genotypes n (%)			Allele frequencies		*p*	He
		CC	CT	TT	C	T		
General population								
- Portugal North	64	21 (32.8)	37 (57.8)	6 (9.4)	0.617	0.383	0.108	0.476
- Portugal Centre	70	25 (35.7)	35 (50.0)	10 (14.3)	0.607	0.393	0.805	0.480
- Portugal South	65	34 (52.3)	27 (41.5)	4 (6.2)	0.731	0.269	0.764	0.396
Total	199	80 (40.2)	99 (49.7)	20 (10.1)	0.651	0.349	0.206	0.455
Symptomatic group	40	16 (40.0)	19 (47.5)	5 (12.5)	0.637	0.363	1.000	0.468

n, number of individuals

p, Exact p-value for the Hardy-Weinberg equilibrium (p significant < 0.05).

He, expected heterozygosity

Exact Test of Sample Differentiation Based on Allele Frequencies: North vs. Centre, p=0.904; North vs. South, p=0.06; Centre vs. South, p=0.05

Table II - Comparison between the results from the breath-hydrogen test and the -13910C>T genotypes in 65 Portuguese individuals.

	Molecular Test		
BH_2T	LNP[a]	LP[b]	
LNP	34	1	35
LP	3	27	30
	37	28	65

[a] LNP= lactase non-persistence: individuals CC_{-13910} homozygous
[b] LNP= lactase persistence: individuals CT_{-13910} heterozygous and TT_{-13910} homozygous