ASSOCIATION OF N ACETYL TRANSFERASE 2 GENE POLYMORPHISMS WITH TYPE 1 DIABETES

Cristian Guja [1], *Sarah Nutland* [2], *Helen Stevens* [2], *Felicity Payne* [2], *John Todd* [2] *and Constantin Ionescu-Tîrgovişte* [1]

[1] 1st Clinic of Diabetes, National Institute of Diabetes, Nutrition and Metabolic Diseases "Prof. N.C. Paulescu", Bucharest, Romania.
[2] Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK.

Abstract

Epidemiological studies suggested an association between dietary nitrates/nitrites and Type 1 diabetes (T1DM), association mediated by the N Acetyl Transferases. Early data indicated a role of N Acetyl Transferase 2 (NAT2) gene in T1DM susceptibility. The aim of our study was to assess the contribution of NAT2 polymorphisms to T1DM genetic susceptibility. For this we typed NAT2 341 T/C, 590 G/A, 803 A/G and 857 G/A polymorphisms by Sequence Specific Primer PCR (SSP-PCR) in 204 Romanian T1DM families. Subsequently we typed the 590 G/A and 803 A/G polymorphisms on another 219 Romanian and 1857 Caucasian families, using Taqman®. Allele transmission to diabetics and unaffected siblings was studied using the Transmission Disequilibrium Test (TDT). We initially found an increased transmission of 590G (60.13%, pTDT=0.0075) and 803G (54.89%, pTDT=0.1) alleles to diabetics. The genotyping of the extra 2076 families did not confirm this association. Thus, on the total of 2280 families, the transmission of 590G allele was 50.55% (pTDT=0.59) while that of 803G allele was 49.25% (pTDT = 0.43). In conclusion, our results indicate that there is no effect of NAT2 gene polymorphisms on T1DM susceptibility.

key words*: Type 1 Diabetes, genetics, N-nitroso compounds, N Acetyl Transferase, Romania*

Background

Type 1 diabetes mellitus (T1DM) is a common autoimmune disease that arises from the specific destruction of insulin secreting pancreatic β cells by autoreactive T lymphocytes [1]. The pathogeny of T1DM is complex and multifactorial, involving the interaction between both genetic and environmental factors [2,3,4]. It is now known that T1DM is a genetically complex disease, caused by multiple susceptibility and protective alleles interacting with each other and with non-genetic factors [5, 6]. The major susceptibility genes are encoded in the HLA region of the Major Histocompatibility Complex (MHC) on chromosome 6p21 [7, 8, 9] and explain approximately 50% of the familial aggregation of T1DM [6]. Other susceptibility loci have been identified in the

5' promoter region of the insulin gene (*INS*) on chromosome 11p15 [10], the *CTLA-4* gene region on chromosome 2q33 [11,12], the *PTPN22* (Lymphoid Tyrosine Phosphatase 22) gene on chromosome 1p13 [13,14], the *IL2RA/CD25* gene region on chromosome 10p15 [15] and the *IFIH1* (Interferon Induced Helicase 1) gene on chromosome 2q24 [16]. Using linkage analysis strategies by whole genome screening, some other regions of the human genome were linked with T1DM [17,18] but none of the putative diabetogenic genes from these regions has been identified yet. However, the study of candidate genes from the regions of interest remains one of the main research priorities.

Some epidemiological studies suggested an association between the dietary intake of nitrates/nitrites (from food and drinking water) and T1DM [19,20]. The association is mediated by their conversion in N-nitroso compounds, reaction that involves the enzymes N Acetyl Transferases [21]. Thus, the N Acetyl Transferases became strong functional candidates for T1DM genetic susceptibility. As a confirmation, our early data suggested a possible association of N Acetyl Transferase 2 (NAT2) gene polymorphisms with T1DM in the Romanian population [22]. The aim of our study was to completely elucidate the influence of NAT2 genetic variants on T1DM genetic susceptibility. For this, we tested four NAT2 Single Nucleotide Polymorphisms (SNP) on 204 Romanian T1DM families and subsequently we tried to replicate our results on another 219 Romanian and 1857 European (or of European descent) Caucasian T1DM families.

Materials and Methods

Subjects. A total of 204 Romanian Type 1 diabetic families were initially genotyped. Data regarding these families were previously reported [23,24]. Subsequently, the analysis was expanded to another 219 simplex and multiplex Romanian families. The ascertainment of these families and collection of biological samples were made according to the same protocol as for the first Romanian collection [23, 24]. This second Romanian family dataset comprised 759 individuals; 335 M (44.13%)/424 F (55.87%) of which 227 type 1 diabetic patients (102 M/125 F) with the onset of disease between ages 1 to 30 years. The median age at the onset of disease was 12.77±5.2 years. There were 532 unaffected individuals (parents and siblings), 233 M (43.79%)/299 F (56.21%) [25].

So overall we analysed a total of 423 Romanian families, with 1515 subjects: 46.6% Males/53.4%Females. The proband cohort included 439 T1DM subjects, with a balanced sex ratio: 208 Males (47.4%) / 231 Females (52.6%), including 430 T1DM children and 9 affected parents. The onset of disease occurred between 9 months and 43 years. The mean age at diabetes onset was 12.6 ± 6.4 years. The vast majority (77.9%) of diabetic probands have disease onset before the age of 17 years while 297 (67.7%) had disease onset before the age of 14 years, fulfilling the EURODIAB ACE Study inclusion criterion.

All the other 1857 families typed in this study were Caucasian, European or of European descent. They comprised of 457 UK multiplex families from Diabetes UK Warren collection [26], 231 US multiplex families from Human Biological Data Interchange [27], 80 Yorkshire simplex families from the

UK, 250 Belfast multiplex/simplex families from Northern Ireland [28] and 839 Finnish multiplex/simplex families [29]. All samples for DNA extraction were collected after informed consent. The protocol for DNA extraction was previously reported [23].

Polymorphisms. We initially selected for typing on the Romanian families 4 coding, non-synonymous, SNP's of the NAT2 gene. These were: 341 T/C (encoding 114 Thr [T] / Ile [I]), 590 G/A (encoding 197 Arg [R] / Gln [Q]), 803 A/G (encoding 268 Lys [K]/Arg [R]) and 857 G/A (encoding 286 Glu [E] / Gly [G]) polymorphisms of N Acetyl Transfearase 2 (NAT2) gene on chromosome 8p21.3-23.1. All these polymorphisms were previously described [30] and can be found in the database of SNP's (http://www.ncbi.nlm.nih.gov/SNP/). The dbSNP id numbers are rs1801280, rs1799930, rs1208 and rs1799931 respectively. The 590 G/A and 803 A/G polymorphisms which showed evidence of association with T1DM were assigned DIL (Diabetes and Inflammation Laboratory) numbers, respectively DIL6768 and DIL6769 (http://www-gene.cimr.cam.ac.uk/ todd/human_data.shtml).

SSP-PCR Genotyping. The 4 NAT2 SNP's were typed on the first 204 Romanian families using the Sequence Specific Primer - SSP-PCR technique as previously described [23,31,32]. Briefly, the technique consisted in specific PCR amplification of individual alleles followed by electrophoresis of PCR products on ethidium bromide stained agarose gels and visualisation of the products with UV illumination. An example is given in Fig. 1. For all alleles an internal control for validation of PCR amplification was used. The sequences of the primers used for PCR amplification were previously reported [33].

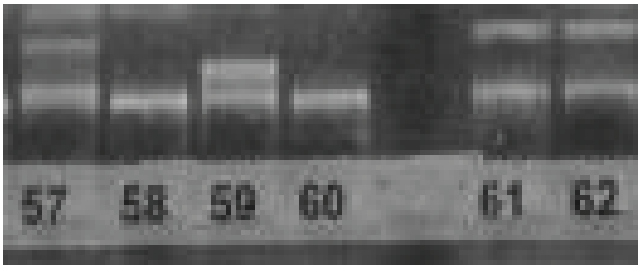

Fig. 1. The polaroid photograph of an electrophoresis gel visualised with UV illumination. PCR products for NAT2 SNP's 341 T/C and 803 A/G (lanes 57; 58 multiplexed), 590 G/A (lanes 59; 60) and 857 G/A (lanes 61; 62).

High throughput SNP genotyping using Taqman. Taqman 5' nuclease assay (Applied Biosystems, Warrington, UK) was used for the genotyping of 590 G/A and 803 A/G polymorphisms on the extra 219 Romanian and 1857 European families. The Taqman allelic discrimination assay is a modern genotyping method which allows the accurate discrimination between the alleles of SNP's, in the same time increasing the throughput and decreasing the costs for large-scale genotyping [34,35].

Taqman probes and primers were designed by the suppliers (Applied Biosystems, Warrington, UK) and their sequence is given in Table 1. For each Taqman assay, the reaction mix consisted of 0.0625 μl Assay Mix, 2.5 μl Universal Master Mix, 0.4375 μl double distilled water and 2 μl genomic DNA at 0.5 ng/μl. The Assay mix and Universal Master Mix were provided by

the producer. All the reactions were performed on 384 well Taqman plates (Applied Biosystems, Warrington, UK) in MJ Research PTC-200 thermal cyclers. The cycling parameters were 10 min at 95°C followed by 40 cycles of 92°C for 15 s and 60°C for 60 s, then 10°C indefinitely. Following amplification, the fluorescent signal from each well is read with an ABI PRISM 7900 HT plate reader, the information being subsequently loaded into the database and converted into final genotypes. An example is given in Fig. 2. All genotyping data were double-scored to minimize error.

Table 1. The sequence of Taqman primers and reporters used for genotyping of 590 G/A and 803 A/G polymorphisms (Genebank Accession Number X14672).

	590 G/A	803 A/G
Forward Primer Sequence	CCTGCCAAAGAAGAAACACCAAAA	CTCTCACTGAGGAAGAGGTTGAAG
Reverse Primer Sequence	GAGACGTCTGCAGGTATGTATTCAT	CACGAGATTTCTCCCCAAGGAA
Reporter 1 (VIC) Sequence	TTGAACCTC**G**AACAAT	AAGTGCTGA**A**AAATA
Reporter 2 (FAM) Sequence	TTGAACCTC**A**AACAAT	AAGTGCTGA**G**AAATA

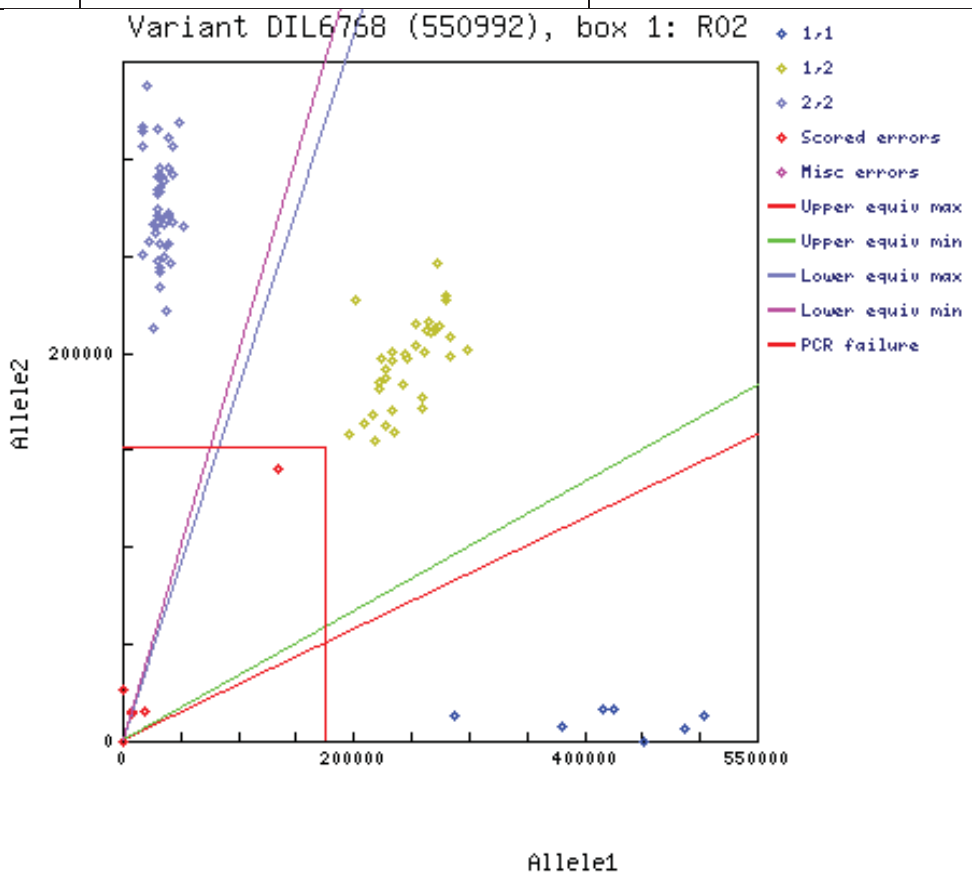

Fig. 2. Genotyping results for 590 G/A polymorphism on one Romanian box (96 individuals). Each point on the graph indicate the genotype for one DNA sample. Allele 1 - 590 A; Allele 2 - 590 G

Statistical analysis. Genotyping data for the first 204 families were initially analysed using the Transmission Disequilibrium Test (TDT) [36] using GAS (Alan Young 1993–1995). The TDT tests the possibility that transmission of alleles from heterozygous parents to affected siblings is not the expected 50%. The TDT relative risk (RR) was calculated by dividing the number of times an allele was transmitted by the number not transmitted.

For all the other families, all statistical analyses were performed in Stata (http://www.stata.com), making use of the Genassoc package (http://www-gene.cimr.cam.ac.uk/clayton/software/).
Allelic association was tested using again the TDT. Genotype relative risk ratio (GTRR) was determined by conditional logistic regression. Robust variance estimates were used for the calculation of *p* values, and 95% CI, in order to correct for non-independence of transmissions within families with more than one affected offspring. Genotyping data were tested for Hardy-Weinberg equilibrium for all the population groups.

Results

The TDT analysis results of the genotyping data on the initial 204 Romanian T1DM families are given in Table 2. From the 4 SNP's tested, we found a significantly increased transmission of the 590G allele to diabetics (60.13% transmission, p_{TDT} = 0.0075) compared to their unaffected siblings (50% transmission). We also found an increased transmission of 803G allele to diabetics (54.89% vs. 52.85%), but with p_{TDT} = 0.1 this was not statistically significant. There was no effect of 341 T/C and 857 G/A SNP's on T1DM risk for this dataset.

Table 2 - TDT results for NAT2 polymorphisms on the 1st Romanian dataset (204) families.

	SNP	T	NT	% T	P_{TDT}
Affected	341 C	104	85	55.03%	0.095
Unafected	341 C	70	55	56.00%	0.11
Affected	590 G	92	61	60.13%	0.0075
Unafected	590 G	51	51	50.00%	0.5
Affected	803 G	101	83	54.89%	0.1
Unafected	803 G	65	58	52.85%	0.29
Affected	857 G	11	12	47.83%	0.5
Unafected	857 G	11	10	52.38%	0.5

T - Transmitted, NT - Not-transmitted, %T - Transmission percentage

In order to confirm our initial results, we typed another 219 Romanian T1DM families only for the 590 G/A and 803 A/G polymorphisms. The results of the TDT analysis for the 2nd Romanian dataset (Table 2 and Table 3) did not confirm our initial findings, showing a non-significant increased transmission of both 590G (54.31%, p_{TDT} = 0.35) and 803G (52.99%, p_{TDT} = 0.51) alleles to diabetics, while 590G allele was also over-transmitted in unaffected siblings (62.33% transmission, p_{TDT} = 0.03). However, the combined analysis of the results for both datasets (423 families) showed a marginally

significant increased transmission of 590G allele to diabetics, with 56.93% transmission and $p_{TDT} = 0.023$ (Table 3).

Table 3. Transmission of the G allele of 590 G/A polymorphism on the whole dataset (2280 families)

Population	No. of families	Disease status	TDT				GTRR
			T	NT	%T	P_{TDT}	p_{GTRR}
Romanian (2nd dataset)	219	affected	63	53	54.31%	0.35	0.62
		unaffected	48	29	62.33%	0.03	0.055
UK	537	affected	344	338	50.44%	0.4	0.67
		unaffected	14	19	42.42%	0.39	0.71
US	231	affected	250	267	48.36%	0.44	0.51
		unaffected	16	12	57.14%	0.48	0.21
Northern Ireland	250	affected	95	105	47.5%	0.49	0.79
		unaffected	47	41	53.41%	0.52	0.41
Finnish	839	affected	300	291	50.76%	0.71	0.68
		unaffected	372	371	50.07%	0.97	1
Romanian (total)	**423**	**affected**	**152**	**115**	**56.93%**	**0.023**	**0.081**
		unaffected	**102**	**81**	**55.73%**	**0.14**	**0.32**
Total	**2280**	**affected**	**1141**	**1116**	**50.55%**	**0.59**	**0.65**
		unaffected	**551**	**524**	**51.26%**	**0.43**	**0.68**

TDT - Transmission Disequilibrium Test, T - Transmitted, NT - Not-transmitted,
%T - Transmission percentage, GTRR - Genotype Relative Risk Ratio

Table 4. Transmission of the G allele of 803 A/G polymorphism on the whole dataset (2280 families)

Population	No. of families	Disease status	TDT				GTRR
			T	NT	%T	P_{TDT}	p_{GTRR}
Romanian (2nd dataset)	219	affected	62	55	52,99	0.51	0.74
		unaffected	43	49	46,74	0.53	0.65
UK	537	affected	331	357	48,11	0.5	0.80
		unaffected	15	15	50,00	1	1
US	231	affected	313	326	48,98	0.6	0.41
		unaffected	13	16	44,83	0.61	0.83
Northern Ireland	250	affected	126	130	49,22	0.8	0.97
		unaffected	54	43	55,67	0.27	0.38
Finnish	839	affected	414	432	48,94	0.54	0.83
		unaffected	564	546	50,81	0.6	0.38
Romanian (total)	**423**	**affected**	**159**	**139**	**53,36**	**0.24**	**0.34**
		unaffected	**111**	**109**	**50,45**	**0.89**	**0.84**
Total	**2280**	**affected**	**1343**	**1384**	**49,25**	**0.43**	**0.69**
		unaffected	**757**	**729**	**50,94**	**0.48**	**0.23**

TDT - Transmission Disequilibrium Test, T - Transmitted, NT - Not-transmitted,
%T - Transmission percentage, GTRR - Genotype Relative Risk Ratio

Finally, the global analysis of the genotyping data for the 2280 families (including the Romanian and an extra 1857 Caucasian families) showed no effect of the 590 G/A and 803 A/G SNP's (Tables 3 and 4). Thus, the transmission of 590G and 803G alleles to diabetics was no different than 50% (50.55% transmission, $p_{TDT} = 0.59$ and 49.25% transmission, $p_{TDT} = 0.69$ respectively).

Discussions

In this study we used a candidate gene approach to ascertain the role of NAT2 genetic variants on the risk for T1DM. susceptibility. Several findings were behind the hypothesis that N Acetyl Transferases could influence the risk of T1DM in humans. First, experimental studies in animal models showed that a diet rich in nitrosamines is associated with a form of diabetes similar with T1DM in humans [37]. Then there are several epidemiological studies which showed that an increased intake of nitrates/nitrites (from food and drinking water) is associated with an increased risk for T1DM [19,20,38]. It was hypothesised that this association is mediated by the conversion of nitrates/nitrites into N-nitroso compounds (including nitrosamines) in the intestinal tract. It was also suggested that nitrates from drinking water are potentially more damaging than those from vegetal foods that contain in the same time important natural inhibitors of the nitrosilation processes (Vitamins A, C and E) [19].

Evidence supporting the hypothesis that N-nitroso compounds could be associated with T1DM risk in humans were provided again by animal data. It is long known that N-nitroso compounds such as streptozotocin and N-nitroso-methyl-urea have a direct toxic effect on the pancreatic beta cells [39]. Subsequently it was shown that streptozotocin (which biochemically is a nitrosamine), if administered repeatedly in low doses, can lead in mice to an immune response directed against the pancreatic beta cells [40] with an insulitis process that progresses towards clinical overt diabetes [41]. This experimental animal model of T1DM (also known as "low dose streptozotocin diabetes") was later reproduced in larger animals, such as minipigs [42]. All these data suggest that, in humans also, repeated exposure to N-nitroso compounds could be associated with insulitis and increased risk for T1DM in genetically susceptible individuals.

The conversion of dietary nitrates/nitrites into N-nitroso compounds in the gut involves (among others) the enzymes N Acetyl Transferases (NAT) [21]. In humans, these enzymes are encoded by the highly homologous genes NAT1 and NAT2, mapped to chromosome 8p21.3-23.1 [43]. In accordance to the level of enzymatic activity, most or perhaps all populations can be stratified into two phenotypes: "rapid acetylators" versus "slow acetylators". It was shown that the highly polymorphic NAT2 gene [30] is responsible for most of this functional variance, "wild type" homozygotes being rapid acetylators while "mutant" hetero and homozygotes being slow acetylators [44]. The "acetylator phenotype" would influence the conversion rate of nitrates/nitrites into N-nitroso compounds. All these data made of NAT2 gene a strong functional candidate for T1DM susceptibility in humans.

We initially chose for NAT2 genotyping the Romanian population, one with the lowest incidences of T1DM in Europe [23].

Previously we tested the Romanian families for *HLA, INS*-VNTR and *CTLA4* in order to validate genetically for Type 1 diabetes this population. [23,24]. The initial genotyping of 204 Romanian families by SSP-PCR for four NAT2 SNP's known to influence the acetylator status [30] suggested an association between 590G allele and T1DM risk and a possible trend for 803G allele. This initial analysis showed no influence on T1DM risk for 3 SNP's of NAT1 gene (data not shown).

Due to the small size of the 1st Romanian dataset, we decided to reconfirm our data by typing another 219 Romanian T1DM families and 1857 families from UK, US, Finland and Northern Ireland. On these families, we typed the 590G/A and 803 A/G polymorphisms using the Taqman® 5' nuclease assay (a reliable and high-throughput genotyping method). Even if the joint analysis of the 423 Romanian families showed a marginal effect of 590G allele (p_{TDT} = 0.023), the analysis of 537 UK, 231 US, 250 Northern Ireland and 839 Finnish families showed no evidence of association of these NAT2 variants with T1DM in any of these populations. Finally, the combined analysis of the 2280 families showed that there is no influence of the 590G/A and 803 A/G on the risk for T1DM in Caucasian populations.

In conclusion, given our negative results, we can state that it is very unlikely that polymorphisms of the NAT2 gene have an influence on the susceptibility for T1DM.

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Correspondence Data:
Dr. Cristian Guja
National Institute of Diabetes, Nutrition and Metabolic Diseases "Prof. N.C. Paulescu"
5-7 I. Movila Street, Bucharest 2, ROMANIA
Tel. 0040 21 210 64 60; Fax. 0040 21 210 22 95
e-mail: cristian.guja@b.astral.ro