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Article

Gene polymorphisms, activation of the Aryl Hydrocarbon Receptor, and DNA damage: a preliminary investigation

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Abstract: 1) Background: We tested whether AHR activation induces DNA damage, whether polymorphisms in genes related to risk of Non-Hodgkin lymphoma are associated with DNA damage, and whether the two conditions do interact with each other.

2) Methods: Our study population included 36 subjects, randomly selected among the population controls participating in a case-control study on lymphoma in Sardinia, Italy, who donated a blood sample. We investigated 47 single nucleotide polymorphisms (SNPs) previously reported to convey risk of lymphoma; the Dual-Glo® Luciferase Assay System to detect activation of the aryl hydrocarbon receptor (AhR) by the serum of study subjects; and the COMET Assay to detect DNA damage.

3) Results: Activation of the aryl hydrocarbon receptor did not increase DNA damage in our study population. On the other hand, the mutant allele **(G)** of **rs1056932/BCL6** increased the occurrence of DNA damage (p=0.045); such association was confirmed among AhR negative, but not AhR positive subjects.

4) Conclusions: We observed excess DNA damage associated with a gene polymorphism, namely **rs1056932/ *BCL6*,** previously reported in association with risk of lymphoma. No increase in DNA damage was associated with AhR activation per se, nor with the other gene polymorphisms we investigated.

Keywords: aryl hydrocarbon receptor; DNA damage; single nucleotide polymorphisms; BCL6; lymphoma.

1. Introduction

Recent studies have shed light on the multifactorial etiology of Non Hodgkin Lymphoma (NHL), including environmental factors and genetic variation [1]. Tetra-chloro-dibenzo-dioxin (TCDD), a known human carcinogen and an etiological agent for NHL[2] strongly activates the aryl hydrocarbon receptor (AhR). AhR, in turn, is a ligand-activated transcription factor (TF) that mediates various cellular responses to xenobiotics, and plays a vital modulatory role in immune cells, including T cell subsets, such as T helper17 and T

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regulatory, antigen presenting cells (APCs) as dendritic cells (DCs), as well as macrophages[3-5], during innate and immune responses.

Once activated, AhR translocates to the cytoplasm, where it forms a heterodimer complex with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), and then into the nucleus, where it binds to a xenobiotic responsive element (XRE) located in the promoter region of the target genes. This complex induces the transcription of plenty of factors connected with different physiological and pathological pathways, which include the metabolism of xenobiotics, cell proliferation and differentiation, and apoptosis [6-9]. Autoimmune diseases, inflammation, cardiovascular diseases, and neoplasms, such as lung, pancreatic and gastric cancer, as well as adenomas, medulloblastoma, and lymphoma have been linked to AHR activation [10]. However, depending on the type of AhR ligand and the specific function of the tissue implicated, either promotion or inhibition of tumor progression can result: for instance, TCDD inhibits the invasiveness of breast cancer, while N-butyl benzyl phtalate enhances its progression [11, 12]. However, a previous study we conducted was inconclusive about the association between the activation of the aryl hydrocarbon receptor (AhR) and risk of NHL [13].

A possible outcome of AHR activation is the production of intermediate metabolites, which can cause oxidative DNA damage. External sources of DNA damage include ionizing radiation, ultraviolet light, alkylating and cross-linking chemotherapeutic agents, cigarette smoke, and others [14, 15]. Different types of DNA lesions, including single and double strand breaks (SSB, DSB), alkylated DNA bases and intra- and inter-strand cross-links would result from exposure to external mutagens. Besides, every cell experiences up to 105 spontaneous DNA lesions per day, which would add up to DNA damage of approximately the same size of that resulting from external agents [16]. DSBs are particularly relevant to the recombination machinery. B and T cells with specialized DNA recombination mechanisms can be highly sensitive to SBs during rearrangement of their immunoglobulin or T-cell-receptor genes. SBs cause DNA damage during mitosis, such as chromosomal aberrations including deletions, chromosomal translocations and aneuploidy-cancerous events, which are associated with risk of lymphoma, leukemia, and other cancers [17].

The role of common gene polymorphisms SNPs involved in lymphomagenesis in modulating the response to mutagenic events triggered by AHR activation is also unclear. These include SNPs in genes implicated in numerous functions and steps in lymphomagenesis, such as DNA repair (XRCC3), cell cycle machinery (MMP9, SELPLG), apoptosis (BCL2, BIM, FAS), cytokines (TNF, LTA, IL6,IL-10), lymphocytes trafficking (CASP8), lymphocyte homeostasis (CASP1), antibody production (IL-1, IL-4), steroidogenesis (CYP19A1, ESR), phagocytosis (FCGR2), antibody maturation (FSF13B, IRF-4), maintaining DNA stability (MGMT), drug toxicity(NAT2), oxidative stress pathway (NOS2A), circadian rhythms (NPAS2), vitamin C uptake (PAIP2), DNA recombination pathway (RAG-1), and pathogen recognition (TLRs) [1, 18-38].

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We tested whether AhR activation and the gene polymorphisms conveying an increased risk of NHL are independently associated with DNA damage, and whether the two conditions interact in causing DNA damage.

2. Results

Our study population included 18 men and 18 women. Mean age was 55 years (*sd* 14.1). Eight subjects were positive for AHR activity. AhR positive subjects were older than AhR negative (AhR positive: mean 63 years, *sd* 6.6; AhR negative: mean 52 years, sd 14.9; *t* test = 2.02, $p = 0.051$), and more frequently male (6/18 men and 2/18 women; $p = 0.11$). Use of medication did not vary by AhR status: 4/8 AhR positive subjects took medication, against 14/28 AhR negative subjects. None of the AhR positive subjects had a recent exposure to chemicals.

2.1. AHR activity versus DNA damage.

After storage at -80°C for 24 - 74 months, cell viability was above the suggested 75% threshold to provide reliable results in 16 out of the 36 samples. To explore the association between AhR activity and DNA damage, we compared the mean DNA percentage in the tail and the mean tail moment by AhR activity: the level of both indicators of DNA damage did not vary by AhR status (Table.1), indicating no association between AhR activity and DNA damage.

Table 1. Mean percent DNA content in the tail A), and tail moment B) by AhR activity.

A

AhR	N	mean DNA% in tail	sd	median DNA% in the tail	Interquartile range	
Neg	11	18.6	9.13	13.3	9.60	17.06
Pos	5	18.0	3.35	10.5	9.81	18.79
All	16	18.4	7.65	12.3	9.59	18.52

B

AhR	N	mean Tail Moment	sd	median Tail Moment	Interquartile range	
Neg	11	26.8	17.17	14.0	10.48	19.69
Pos	5	26.5	6.67	12.8	10.13	19.32
All	16	26.7	14.44	13.5	9.96	19.60

2.2. SNPs versus DNA damage.

As shown in Table 2, only the mutant allele (G) of rs1056932/BCL6 showed an association with DNA damage, when we considered DNA percent in the tail above the median (p=0.045), but not when we considered tail moment (p=0.399; not shown in the Table). We observed some indication of an association also for the mutant allele (A) of rs1800871/IL10

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(*p*=0.140), and the mutant allele (C) of rs4934436 (FAS) (*p*=0.156), also not confirmed by tail moment above the median (*p*=0.872 and *p*=0.827, respectively). It is interesting to notice that the association with the BCL6 polymorphism and the suggestive finding for the FAS mutant allele depended upon the AhR activation status, as both were confirmed among the AhR negative subjects (*p* = 0.025, and *p*=0.114, respectively), while there was no association among the AhR positive subject. None of the other SNP-DNA damage results varied by AhR activation status.

Table 2. Association (*p*-values) between the selected gene polymorphisms and DNA damage as detected with the Comet assay. AHR results were divided in two groups: positive and negative.

SNPs	MAF	Obs/exp	*p*-value for MAF	*p*-value for DNA damage		
				All	AhR +	AhR -
rs737865 / COMT	G	1.00/ 0.23	4.06^{-10}	-	-	-
rs36686/ B3GNT3	G	0.26/ 0.26	0.948	0.752	0.739	1.0
rs3132453/ BAT2	T	0.03/ 0.03	0.913	0.361	0/5	0.317
rs2849377/ BCL2	T	0.29/0.09	1.17^{-8}	-	-	-
rs3789068/ BCL2L11 (BIM)	G	0.72/0.35	9.36^{-8}	-	-	-
rs1056932/ BCL6	G	0.42/ 0.43	0.863	**0.045**	0.739	**0.025**
rs3172469/ BCL6	G	0.01/ 0.32	6.40^{-6}	-	-	-
rs3769821/ CASP8	G	0.13/ 0.44	5.59^{-5}	-	-	-
rs6736233/ CASP8	C	0.06/ 0.19	0.009	-	-	-
rs16994592/ CD70 (TNFSF7)	C	1.00/ 0.08	4.6^{-136}	-	-	-
rs1785882/ COP1 (CASP1)	T	0.61/ 0.36	0.0004	-	-	-
rs1870049/ CYP19A1	C	0.01/ 0.28	1.98^{-5}	-	-	-
rs3020314/ ESR1	T	0.97/ 0.38	3.58^{-16}	-	-	-
rs4934436/ FAS	C	0.28/ 0.29	0.847	0.156	0.739	0.114
rs1801274/ FCGR2A	G	0.68/ 0.44	0.002	-	-	-
rs2243248/ IL4	G	0.04/ 0.10	0.118	0/11	0/5	0/6
rs1800797/ IL6	A	0.14/ 0.14	0.980	0.892	0/5	1.0
rs1800871/ IL10	A	0.36/ 0.43	0.373	0.140	0.739	0.456
rs1800872/ IL10	T	0.49/ 0.43	0.468	0.361	0.414	0.317
rs1800890/ IL10	T	0.50/ 0.21	7.88^{-8}	-	-	-
rs1800896/ IL10	C	0.47/ 0.27	0.001	-	-	-
rs485497/ IL12A	A	0.71/ 0.49	0.0003	-	-	-
rs12211228/ IRF-4	C	0.21/ 0.12	0.030	-	-	-
rs3212038/ XRCC3	G	0.34/ 0.28	0.364	0.752	0.414	1.0
rs2308321/ MGMT	G	0.24/ 0.06	1.06^{-9}	-	-	-
rs1041983/ NAT2	T	0.46/ 0.39	0.353	0.399	0.182	1.0
rs1799929/ NAT2	T	0.58/ 0.27	3.11^{-7}	-	-	-
rs1799931/ NAT2	T	0.04/ 0.08	5.7^{-160}	-	-	-
rs2297518/ NOS2A	A	0.04/ 0.17	3.43^{-59}	-	-	-

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rs2305160/ NPAS2	A	0.21/ 0.20	3.04^{-29}	-	-	-
rs6596473/ PAIP2 (SLC23A1)	G	0.88/ 0.47	5.37^{-07}	-	-	-
rs2227973/ RAG1	G	0.82/ 0.25	0.239	0.361	0.414	0/6
rs7300972/ SELPLG	C	0.00/ 0.11	0.005	-	-	-
rs 17576/ SLC23A2 (MMP9)	G	0.22/ 0.46	0.003	-	-	-
rs699517/ ENOSF1	T	0.42/ 0.49	0.317	0.399	0.739	0.456
rs1921310/ TANK	G	0.40/ 0.06	1.61^{-32}	-	-	-
rs4833103/ TLR1	A	0.38/ 0.14	9.86^{-8}	-	-	-
rs10008492/ TLR10	C	0.19/ 0.16	0.465	0.317	0.221	0/6
rs2582869/TNFSF13B	A	0.04/ 0.45	1.28^{-10}	-	-	-
rs886441/ VDR	G	0.25/ 0.23	0.723	0.409	0.739	0.317
rs3819545/ VDR	G	0.64/ 0.40	0.001	-	-	-
rs2239186/ VDR	G	0.65/ 0.20	8.63^{-18}	-	-	-
rs1799724/ TNF	T	0.18/ 0.09	0.010	-	-	-
rs909253/ LTA	G	0.29/ 0.39	0.182	0.409	0.739	0.317
rs361525/ TNF G-238A	T	0.25/ 0.10	5.34^{-69}	-	-	-
rs1800629/ TNF-308α	A	0.04/ 0.09	3.4^{-133}	-	-	-
rs2239704/ LTA	A	0.32/ 0.35	0.661	0.828	0.739	0.317

3. Discussion

Our results suggest that AhR activity per se would not cause direct DNA damage, though DNA damage in AHR negative samples was higher than AHR positive ones. Many different ligands, external and internal are known to act through AhR activation; it is likely that the type of response following AhR activation would change as a function of the type of ligand, directing the link to the xenobiotic-responsive-element of specific metabolic genes, which in turn would activate or not specific xenobiotics capable of causing DNA damage.

We observed a link between the (G) allele of the rs1056932 SNP of *BCL6*, a synonymous substitution, and DNA damage, which was prominent in AhR negative subject, and did not show up among AhR positive subjects. This finding emerged when using the DNA percentage in the tail as the measure of DNA damage. BCL6 is normally expressed in B and T cells of the lymph node germinal center, in other lymphoid tissues, in skeletal muscle cells, nasopharynx, bronchus, testis and in keratinocytes [39]. Functionally, it is a transcription factor which leads the differentiation of naive helper T cells into follicular Helper T cells (TFH cells)[40]. It is thought that for germinal center B-cells to tolerate DNA damage, BCL6 inhibits the expression of genes encoding regulators of DNA damage response, such as *TP53*, *ATR*, *CHEK1*, *GADD45A*, *PC4*, *P21* and *P27KIP1*; among the above, the interaction between BCL6 and TP53 is very important as Tp53 is a vital key in regulating apoptosis [41-44]. Studies showed that BCL6 functions normally to suppress p53-mediated apoptosis of GC B cells in response to DNA damage during the GC reaction. Constitutive expression of BCL6 might decrease the p53-mediated apoptotic response to DNA damage, promoting persistence of malignant clones[45].

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The rs1056932 SNP we investigated is located in exon 6 of BCL6, that alters a potential binding site for an exonic splicing enhancer, which was previously shown to be related to NHL risk [46]. The novel finding in our study is that this allele apparently increases the probability of DNA damage, which is a common sign of tumorigenesis in different cancers and diseases. Besides, the effect seems to be related to AhR activity, and specifically it shows up in subjects negative for AHR activation, but not in those AHR positive. Recently it has been demonstrated that the AHR/ARNT pathway through MEF2B (myocyte enhancer-binding factor 2) regulates BCL6 [47]. In our study, we observed a higher prevalence of the *BCL6* mutant allele among AHR negative study subjects; the level of DNA damage in these subjects was also more elevated. However, we still do not know the exact mechanism underlying the effects associated with this polymorphism.

FAS(CD95)

Although we could not did not observe a correlation between the C allele of rs4934436/*FAS* (*p*-value=0.156) and DNA damage, we still think our result would confirm an effect, previously shown in connection with risk of NHL and some of its subtypes[31]. FAS(CD95) is a proapoptotic protein expressed in GCs (germinal center). FAS ligand cross-links the transmembrane FAS death receptor, leading to the assembly of a death-inducing signaling complex and triggering caspase-mediated apoptosis. *FAS* mutations likely act in a dominant-negative manner, destabilizing trimeric FAS receptors and they have been reported in up to 20% of DLBCL [48].

Our results must be interpreted with caution, due to the exploratory nature, and the small size of our study. The deviation from the expected minor allele frequency for two thirds of the SNPs we tested, might be a consequence of the small sample size; however, we cannot discard the genetic peculiarities of the Sardinian population as a contributing factor, as all the participating subjects were born and resident in Sardinia. On the other hand, as far as we are aware of, no studies have assessed the distribution of the 47 SNPs we explored among the Sardinian population nor the background level of DNA damage in a general population. Although no conclusive inference can be drawn, extension of the study population and replication of our preliminary findings in larger data sets are warranted.

We also conducted multiple comparisons, which might have originated significant findings simply by chance. We did not apply any correction to the α error threshold we set to reject the null hypothesis. This was an exploratory, preliminary study meant to raise hypotheses; therefore, we allowed the few suggestive findings to emerge, and we discussed them in the light of the existing literature to foster further exploration in larger data sets. If our results would be confirmed, research should be addressed to investigate the function of the BCL6 protein expressed by the mutated gene and its interaction with the genes encoding regulators of DNA damage response, and particularly TP53.

4. Materials and Methods

Study samples

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We randomly selected blood cell samples of 36 population controls who participated in a case-control study of lymphoma in southern Sardinia, Italy, in 2007-15. Controls were a random sample of the resident population in the study area, frequency matched to the incident lymphoma cases by gender, and 5-year age group in the 20-75 years age range. Following the Helsinki protocol for studies involving human subjects, before participation, all study subjects signed a consent form for the use of their biological samples for scientific purposes, which explained the procedure to secure the safe storage of personal data, and sample identity. The study protocol included an in-person interview, conducted by trained interviewers at the residence of the study subject, gathering information on demographics, lifestyle habits (smoking, alcohol, diet, leisure time and physical activity), health history, and a detailed occupational history. At the end of the interview, subjects were requested to donate a 40 ml blood sample to investigate genetic and epigenetic determinants of disease, as well as biomarkers of environmental exposures of interest.

Gene polymorphisms

Five hundred microliters serum aliquots were preserved at -80°C; the buffy coat was preserved with DMSO and foetal calf and stored in liquid nitrogen. We used the DNA extraction 500 arrow® Kit (DiaSorin Irland Ltd) to extract DNA from blood, and the Qubit® Fluorometer (Invitrogen™) to quantify it. We selected 47 single nucleotide polymorphisms (SNPs) previously reported to convey risk of lymphoma and its subtypes, based on published reports (Table 3). We checked the functional information on the SNPs available at the SNP databases https://www.ncbi.nlm.nih.gov/snp/, and https://www.ensembl.org/index.html.

Table 3 Single nucleotide polymorphisms selected for their function and their role in lymphomagenesis.

SNP	***Genes***	***Polymorphic Allele /MAF***	***Chromosome***	***SNP's function***	***Protein's function***
rs737865	*COMT*	G / 0.23	22	intron	one of several enzymes that degrades Catecholamines
rs36686	*B3GNT3*	G / 0.26	19 P	missense H328R	lymphocyte trafficking and migration
rs3132453	*BAT2*	T / 0.03	6p	intron, V 1883 L	Part of a cluster of genes (BAT1-BAT5), function is un-known but it has been said that some of its member are involved in DNA-damage-induced apoptosis.
rs2849377	*BCL2*	T / 0.09	18	Intron, protein coding	Apoptosis
rs3789068	*BCL2L11 (BIM)*	G / 0.35	2	Intron, protein coding	BCL-2 interacting mediator of cell death or BIM
rs1056932	*BCL6*	G / 0.43	3q	synonymous	Controls germinal-center formation and T-cell–dependent immune responses.
rs3172469	*BCL6*	G / 0.32	3	intron	
rs3769821	*CASP8*	G / 0.44	2	Intron	
rs6736233	*CASP8*	C / 0.19	2	Intron	regulating lymphocyte homeostasis, NF-kB

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					activation, and differentiation of monocytes into macrophages,
rs16994592	*CD70 (TNFSF7)*	C / 0.077	19	Intron	
rs1785882	*COP1 (CASP1)*	T / 0.36	11	intron	Caspases 1, 4, and 5 play a key role in maturation of pro-inflammatory cytokines in cells infected by certain pathogens, and caspase 1 is the most efficient caspase in the process.
rs1870049	*CYP19A1*	C / 0.28	15	intron	Steroidogenesis
rs3020314	*ESR1*	T / 0.38	6	intron	Estrogen receptor alpha
rs4934436	*FAS*	C / 0.29	10	unknown	Apoptosis
rs1801274	*FCGR2A*	G / 0.44	1	Missense, H 165 R	Receptor for Fc fragment of IgG, low-affinity IIa (CD32) increases the affinity of binding to IgG2 and increases phagocytosis of IgG2 Opsonin.
rs2243248	*IL4*	G / 0.1	5q	5' near gene	B cells switching to IgE antibody production and maturation of helper T cells to a Th2 phenotype
rs1800797	*IL6*	A / 0.14	7p	5' near gene	A pleotropic Cytokine which regulates immune mechanisms by initiating the acute phase of response
rs1800871	*IL10*	A / 0.43	1	5' near gene	A cytokine involved in immune regulation through prominent anti-inflammatory and immune-regulatory activities
rs1800872	*IL10*	T / 0.43	1	unknown	
rs1800890	*IL10*	T / 0.21	1	unknown	
rs1800896	*IL10*	C / 0.27	1	Intergenic	
rs485497	*IL12A*	A / 0.49	3	Intergenic (regulatory function), Transition Substitution	plays a central role in bridging the cellular and humoral pathways of innate resistance and antigen-specific adaptive immune responses
rs12211228	*IRF-4*	C / 0.12	6	3' UTR, Transversion Substitution, Intragenic	IRF4 is a B-cell proliferation and differentiation protein essential for class switch recognition and antibody maturation
rs3212038	*XRCC3*	G / 0.28	14	Intron	DNA repairing
rs2308321	*MGMT*	G / 0.06	10	Missense, 5' near gene I 143 V. Transition Substitution, Intragenic	maintaining DNA stability

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rs1041983	*NAT2*	T / 0.39	8	synonyms Transition Substitution, Intragenic	both activate and deactivate aryl-amine and hydrazine drugs and carcinogens. Contribute to drug toxicity
rs1799929	*NAT2*	T / 0.27	8	Synonyms, L 161 L Transition Substitution, Intragenic	
rs1799931	*NAT2*	T / 0.077	8	Missense Transition Substitution, Intragenic	
rs2297518	*NOS2A*	A / 0.17	17p	Missense, SER 608 LEU Transition Substitution, Intragenic	Oxidative stress pathway
rs2305160	*NPAS2*	A / 0.20	2	Missense, Ala 394 Thr Transition Substitution, Intragenic	Circadian Protein
rs6596473	*PAIP2 (SLC23A1)*	G / 0.47	5	Intron, Transversion Substitution, Intragenic	VIT.C uptake
rs2227973	*RAG1*	G / 0.25	11	Missense, K 820 R Transition Substitution, Intragenic	Recombination activating protein
rs730097	*SELPLG*	C / 0.11	12	Missense, M 264 V Transition Substitution, Intragenic	encodes a ligand for P-selectin (PSGL-1), which is important for tethering and rolling of leukocytes to endothelial cells and platelets and also may activate Integrin in neutrophils.
rs17576	*SLC23A2 (MMP9)*	G / 0.46	20	Missense Mutation, Transition Substitution, Intragenic	Cell cycle signaling
rs699517	*ENOSF1*	T / 0.49	18	unknown	Down regulating thymidylate synthase
rs1921310	*TANK*	G / 0.06	2	Intron, Transition Substitution, Intragenic	TANK inhibits TRAF function (the TNF receptor-associated factor family) play indirect role in mediating autoimmunity
rs4833103	*TLR1*	A / 0.14	4P	4p Transversion	TLRs are Connected to recognizing a broad

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				Substitution, Intergenic / Intragenic	spectrum of pathogen ligands.
rs10008492	*TLR10*	C / 0.16	4	Transition Substitution, Intergenic /Intragenic	
rs2582869	*TNFSF13B*	A / 0.45	13	intron Transition Substitution, Intergenic / Intragenic	TNFSF13B (encodes the cytokine BAFF) and its main receptor BAFF-R (encoded by TNFRSF13C) play a central role in peripheral B-cell survival and maturation, including inducing class switch recombination
rs886441	*VDR*	G / 0.23	12	intron	Vitamin D receptor
rs3819545	*VDR*	G / 0.4	12	intron	
rs2239186	*VDR*	G / 0.2	12	intron	
rs1799724	*TNF*	T / 0.09	6	Intron, Transition Substitution, Intragenic	Tumor necrosis factor
rs909253	*LTA*	G / 0.39	6	Intron, Transition Substitution, Intragenic	a cytokine produced by lymphocytes; mediates a large variety of inflammatory, immunostimulatory, and antiviral responses
rs361525	*TNF G-238A*	T / 0.099	6	5' near gene	
rs1800629	*TNF-308α*	A / 0.09	6	6P Intron, Transition Substitution, Intragenic	TNF-a is a cytokine mainly secreted by macrophages; involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
rs2239704	*LTA*	A / 0.35	6p	Intron, UTR 5, C 91 A, Intragenic	

Before proceeding with allelic discrimination, we diluted DNA with ultra-pure water up to a 2ng/µl concentration. Primers and probes were purchased from ThermoFisher Scientific (ThermoFisher Scientific, Waltham, MA, USA), and prepared in 20 plates (98 wells). We added 10 µl of Taq Polymerase enzyme, and 10 µl of DNA (2ng/µl) to each well to reach the final DNA concentration of 1 ng/µl. The plates were run for up to 42 cycles (stage 1: denaturation at 95° C for 5 min without repeat; stage 2: denaturation at 95° C for 15 s, and primer attachment and elongation for 1 min at 60°) in a 7300 Real-time PCR TaqMan™ platform (Applied Biosystems, Thermofisher Scientific, Waltham, MA, U.S.A.).

AHR activation

To test AhR activation, we used a Dual-Glo® Luciferase Assay System (Promega Corporation, Madison, WI, U.S.A.), which is based on the response of hepatocellular

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carcinoma (Hep G2) cells [HEPG2] (ATCC® HB8065™, Manassas, VA, U.S.A), purchased and received in January 2014. Cells were incubated in 5% CO_2 at the temperature of 37°C under regularly controlled conditions, in an incubator with a regular maintenance program, and maintained in D-minimum essential medium (D-MEM) (Sigma-Aldrich, Milan, Italy), supplemented with 10% (v/v) heat inactivated FBS with 1% penicillin/streptomycin and 1% L-glutamine, according to the manufacturer instructions. Cells viability was 98%, and it was checked by optical microscopy, with no evidence of deviation from the expected growth process or Mycoplasma contamination. Before use in the experiment, cells underwent three passages: 1. after thawing, we kept them for 24 hours in a 25mm flask; 2. then we made aliquots in 75mm flasks; and 3. after two days, HepG2 cells were seeded at a density of 1 x 10^4 cells/well in 24-well plates. After 24 hour, following the manufacturer instructions, the HepG2 cells medium was replaced with a fresh medium and the cells were transfected using the FuGENE HD Transfection Reagent (Promega Corporation, U.S.A.) with plasmids containing the reporter system, the pGL4.43 [luc2p/XRE/HYGRO] vector (1 µg/ml), which carried the XRE enhancer sequence for the luciferase gene, and the pGL4.73 [hRLucSV40] vector (100 ng/ml), an internal control of the efficacy of transfection with a strong promoter, such as the SV40, that drives transcription of the reporter gene. Twenty four hours later, the medium was replaced with a fresh medium Opti-MEM (Life Technologies, Paisley, UK) and the cells were exposed to the serum sample 10% (v/v) of study subjects, heat-inactivated by incubation at 65° for 30 min, and tetra-chloro-dibenzo-dioxin (TCDD, Sigma-Aldrich, Italy) was added to the cells at increasing concentrations in the 10^{-12} -10^{-8} M range. The lowest TCDD dose capable of activating the system was detected at 1* 10^{-9} M; such concentration was used as the positive standard. Three wells were designated to one treatment condition.

Following AhR induction, we measured the presence of the luciferase protein in the 20 µl of cell lysate with a luminometer. The response to the positive standard and to the activating molecules in the serum samples of study subjects was detected with 100 µl Stop &Glo® Reagent using a luminescence reader (Victor x5 Perkin Helmer, Waltham MA, USA). The luminescence is translated into a numeric value, directly correlated with the luciferase expression, which in turn is proportional to the AhR activity. Such reading is then referred to the internal control of transfection efficacy (the vector containing the non-induced luciferase gene), the positive control (10^{-9} M TCDD), and the negative control (the HepG2 cells without the serum sample). All mandatory laboratory health and safety procedures had been complied with in conducting the above described assays.

Comet assay

We determined DNA damage in the samples of study subjects with the COMET assay using the COMET assay (IKZUS ENVIRONMENT® Kit, Alessandria, Italy). Before conducting the assay, following the manufacturer instructions, we checked cell viability and restricted the test to cell samples with > 75% viability in order to provide reliable results. We thawed cells at 37°C, and immediately added to 10 ml cold, free $Mg^{2+}$and Ca^{2+} PBS, centrifuged (1600 RPM, 5 min, 4°C), and re-suspended them in 1 ml fresh and cold $PBS^{-/-}$ on ice for 5 min. We used trypan blue to check cell viability: nonvital cells are identified in optical

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microscopy by their staining, made possible by the permeability of their cell membrane. Seventeen samples matched the requirement. These samples had been preserved on average for 1363 days (standard deviation, *sd*, 496).

To prepare the samples, we used a lysis buffer (Lysis Sol); Low Melting Point Agarose (LMPA); disodium EDTA, 0.5 M; a neutral buffer (Neutral Sol); FLUOPlus DNA stain®, a new nuclear fluorophore, with a high chemical affinity to single strand (ssDNA) and double strand break DNA (dsDNA), and a virtually absent background signal; bidistilled water; sodium hydroxide in pellets. We attained a number of 20000 cells per slide, as counted in a Neubauer chamber, layered with LMPA strata, with the Singh sandwich technique. Cell lysis was obtained after one hour in the dark at 4°C, followed by unwinding at pH 13, which allows to determine the single and double strand damage by migration in Single Cell Gel Electrophoresis (SCGE) of the negatively charged DNA towards the positive pole, proportionally to its extent of damage and with a speed inversely proportional to the fragment size. After return to neutral Ph, we used FLUOPlus® (excitation wave length: 490 nm; emission wave length: 535 nm) to stain DNA; FLOUplus® linked to DNA emits a green fluorescence, which can be detected after 48 hours on a fluorescence microscope at 20-40x, connected to COMET Assay IV® (Perceptive Instruments Ltd, Bury St Edmunds, United Kingdom) for image analysis. For each slide, we selected a random duplicate sample of 20-25 cells for the calculation of the statistical parameters, including Head Length; Tail Length (L); fluorescence intensity as % Head DNA and % Tail DNA (I); Tail Moment (L x I) and average Tail Moment; and Damage Index (DI).

We calculated the binomial probability distribution to assess the correspondence between the MAF of the individual SNPs in our study with that reported in the SNPs data bases. Sixteen SNPs out of the 47 we assessed (34%) the MAF did not deviate from the expectation based the above-mentioned SNP databases. We categorized two Comet Assay parameters, DNA percentage in the tail and tail moment, into whether below or above the median, and we used a chi-square test to test the chance probability of the association between the mutant alleles of these 16 SNPs and DNA damage indicators, as derived from the values of the two Comet Assay parameters above the median, overall and by AhR activity in 11 study subjects with complete data. We rejected chance when the probability was below 5%. The analysis was conducted with SPSS version 20®.

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