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Final Progress Report – 2006-2012

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PI: Dr. David Bernstein, Professor
University of Cincinnati
College of Medicine
Internal Medicine, Department of Allergy and Immunology
3255 Eden Ave
Cincinnati OH 45267-0563
(513) 558-4701
David.bernstein@uc.edu

Institution: University of Cincinnati
Sponsored Research Services
University Hall, Suite 530
51 Goodman Drive
P.O. Box 210222
Cincinnati OH 45221-0222
(513) 556-4358

Project Title: Genetic Susceptibility for Occupational Asthma

Co-investigators:
Campo, Paloma, University of Cincinnati (2006-2008)
Chakraborty, Ranajit (2006-2009), University of Cincinnati
Hershey, Gurjit Khurana, Cincinnati Children's Hospital Medical Center
Lummus, Zana L., University of Cincinnati
Wang, Ning (2006-2007), Cincinnati Children's Hospital Medical Center

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List of Terms and Abbreviations

AJC	Adherens junctional complex
CD4	Cluster differentiation cell membrane antigen 4
CD8	Cluster differentiation cell membrane antigen 8
CD14	Cluster differentiation cell membrane antigen 14
CTL	Cytotoxic lymphocyte
CTTNNA3	Gene coding for α-catenin
DA	Diisocyanate induced asthma
EPHX1	Microsomal epoxy hydrolase
FEV1	Forced expiratory volume in 1 sec
FVC	Forced vital capacity
GSTM1	Glutathione transferase M1
GSTM3	Glutathione transferase M3
GSTP1	Glutathione transferase P1
GWAS	Genome wide association study
HDI	Hexamethylene diisocyanate
HLA	Human leucocyte antigen
HLA-B, HLA-E	MHC Class I genes
HLA-DOA, HLA-DQH2, HLA-DBP1	MHC Class II genes
IL-4Ra	Interleukin 4 receptor α
IL-5	Interleukin 5
IL-8	Interleukin 8
IL-13	Interleukin 13
LTB4	Leukotriene B4
MDI	Methyl diphenyl diisocyanate
MMP-9	Matrix metallopeptidase 9
NAT1, NAT2	N-acetyltransferase enzymes
OA	Occupational asthma
PC20	Dose of methacholine (mg/ml) for 20% drop in lung function
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SOD2	Mitochrondrial superoxide dismutase
Th2	T helper type 2 lymphocyte
TDI	Toluene diisocyanate

Title: Genetic Susceptibility for Occupational Asthma

Investigator: David I. Bernstein, University of Cincinnati, College of Medicine, Department of Internal Medicine, Division of Immunology and Allergy, Cincinnati, OH 45267-0563 (Email: Bernstdd@ucmail.uc.edu).
Affiliation: University of Cincinnati College of Medicine
State: OH
Telephone: (513) 558-3612

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Final Report Abstract

Diisocyanates are highly reactive chemicals that cause occupational asthma (OA) in 5-15% of exposed workers. Since new cases are not entirely preventable, there is a need to find markers of susceptibility to identify those worker groups at risk for OA, caused by diisocyanates (DA) to further guide risk assessment strategies.

The clinical manifestations of DA are similar to those found in non-occupational asthma, including airway hyperresponsiveness, airway remodeling, as well as a unique ability to illicit isolated late phase responses, with infiltration of eosinophils, basophils, and/or neutrophils. Evidence suggesting immune mechanisms underlying DA susceptibility include a latency period of exposure preceding sensitization, and elicitation of asthmatic responses by sub-irritant levels of chemical. However, unlike OA induced by high molecular weight sensitizers, no sensitive and specific antibody markers of DA have yet been identified. Due to the inherent toxic nature of these chemicals, pathogenesis of DA is likely to be multifactorial in nature, involving innate and acquired immune mechanisms, oxidative stress, and impaired epithelial cell barrier function.

Our approach has been to investigate candidate genes in exposed workers for polymorphisms associated with susceptibility to DA. Using DNA from workers at risk for OA, recruited from occupational respiratory clinics in Canada and Spain, , we have identified Th2 cytokine gene polymorphisms, antioxidant enzyme gene polymorphisms, HLA class I and II alleles, and α-catenin (CTNNA3) SNP variants significantly associated with the DA phenotype.

In the current study, we confirmed results of a previous study in which we identified single nucleotide polymorphisms (SNPs) in the IL-4Ra, IL-13, and CD-14 genes that were statistically associated with diisocyanate asthma in workers exposed to hexamethylene diisocyanate (HDI), using an expanded number of symptomatic workers (DA+ and DA-, confirmed by SIC),as well as a new group of asymptomatic controls (AWs) for comparison.

Further studies emanating from this grant yielded finding of significant associations between novel MHC Class I (HLA-E, HLA-B) and Class II (HLA-DOA, HLA-DQA2 and HLA-DBP1) SNPs and DA.

Candidate gene association studies were then extended to investigate SNPs of genes were selected based on their functional role in oxidative stress and inflammation. Anti-oxidant enzyme genes including: glutathione S-transferase [GSTM1 (null), GSTM3 (3 SNPs), GSTT1 (null) and GSTP1 (Ile-Val) SNPs]; mitochondrial superoxide dismutase (SOD2; Ala-Val SNP); and eight epoxide hydrolase (EPHX1) SNPs. Using logistic regression modeling, SOD2 rs4880, EPHX1 2740171 and GSTP1 rs1695 variants are significantly associated with DA. After adjustment for confounding variables, variants of GSTM1, GSTT1, GSTP1, EPHX1 and GSTM3 genes also showed significant positive or negative associations with DA.

Finally, we were able to replicate the findings from a GWAS study of Korean DA workers that demonstrated an association between two closely linked CTNNA3 gene SNPs and DA, in our European population of DA workers. As in the Korean study, highly linked CTNNA3 rs7088181 and rs10762058 SNPs were significantly associated with DA+ when compared to AWs. The CTNNA3 gene codes for α-T-catenin, a cytoplasmic anchorage protein in the epithelial adherens junctional complex (AJC), which is critical for maintaining epithelial cell-cell adhesion and epithelial barrier function. These finding served as the basis for our renewal proposal in which we plan to perform next generation DNA sequences of informative loci in the AJC and to identify functional variants.

The genetic risk factors identified can contribute to future intervention studies to reduce the occurrence of DA in workers.

Section 1: Significant (Key) Findings

Specific Aim #1. Expand ongoing case-control studies in workers referred for evaluation due to reactive chemicals to include associations of an expanded panel of genetic polymorphisms.

1. IL-4Ra, IL-13, and CD14 gene polymorphisms are statistically associated with susceptibility to diisocyanate induced asthma.

   The finding of genetic variants of the IL-4 receptor α and IL-13 as a risk factor for DA establishes a role for immunological pathogenetic mechanisms in DA. Respiratory sensitization occurs by inhalation of a sensitizer, uptake by antigen-presenting cells such as dendritic cells that process antigens, and migration of these cells to regional lymph nodes where antigen is presented to $CD4^+$ T helper (T_H) cells that initiate an immune response. The nature of the immune response is influenced by the cytokine milieu at the site of lymphocyte stimulation. Depending on host factors and the antigenic epitopes, T_H cells differentiate into subpopulations of effector cells that produce different cytokines. T_H 2 cells produce 3 cytokines, IL-4, IL-5, and IL-13, shown to critically influence asthma pathogenesis in mouse models, and the levels of all these are increased in asthmatic patients. The finding that a CD14 polymorphism can also modify susceptibility to DA suggests that innate immune mechanisms are also involved in DA pathogenesis. CD14 is a component of the innate immune system and was the first described pattern recognition receptor. It is expressed mainly on macrophages, but CD14+ monocytes can differentiate into a host of different cells, including dendritic cells, a differentiation pathway encouraged by cytokines, including IL-4.
2. A role for HLA immunoregulatory mechanisms underlying DA pathogenesis was established. SNPs mapping to both HLA Class I (HLA-E, HLA-B) and HLA Class II (HLA-DOA, HLA-DQA2, and HLA-DPB1) genes showed associations with altered risk of developing DA. This finding suggests that both cellular and humoral immune responses are involved in DA. Antigen epitopes bound to Class I molecules are recognized by cytotoxic T cells ($CD8^+$) , leading to cell death, while antigen epitopes bound to Class II molecules are recognized by T helper cells, leading to antibody formation and phagocytosis.
3. Anti-oxidant enzyme gene variants, including mitochondrial superoxide dismutase (SOD2) and epoxide hydrolase (EPHX1) and glutathione S-transferase (GSTP1) variants were shown to be associated with susceptibility to DA. This further suggests a role for oxidative stress and inflammation in the pathogenesis of DA and may be utilized in future risk assessment strategies of workers exposed to diisocyanates.
4. We demonstrated an association between two closely inked CTNNA3 gene SNPs and DA in our workers of European descent, in a replication study of a GWAS performed in Korean DA workers. The demonstration that variants of CTNNA3 are associated with DA in two geographically unique populations gives increased importance for this gene and dysfunctional AJC proteins as possible susceptibility markers of DA.

Translation of Findings

Currently the treatment for subjects diagnosed with DA is supportive asthma therapies and cessation of exposure. It appears likely that a reduction in risk of DA could be achieved by genetic screening for gene variants associated with DA. This will require further research to determine a functional role for the aforementioned disease associated gene polymorphisms. In particular, gene sequencing and identification of linked or tagging SNPs associated with the latter DA associated variants may reveal these or other SNPs that are functionally relevant to the pathogenesis of DA. A profile of disease associated functional variants may be used to identify high risk workers. This information could be used to direct special prevention strategies in high risk subgroups of isocyanate workers.

Outcomes/Impact

1) **Potential outcomes** (findings that could impact workplace risk if used)

These studies were directed at meeting the goals and intent of the NIOSH programmatic Research to Practice (r2p) initiative which calls for extramural NIOSH-funded proposals that transfer or translate innovative research findings to effective prevention practices which can be adapted to workplace practices that will reduce illness and injury. Consistent with the r2p initiative, our results have identified & validated single or multiple SNPs that in the future can be utilized as susceptibility markers for OA caused by reactive chemical agents and which may identify asymptomatic workers at greatest risk for development of airways inflammatory responses and, ultimately, occupational asthma. Further, our studies are likely to have a major impact on human health in that, for the first time, a large DNA databank of extremely well phenotyped OA cases and control subjects from a variety of background populations has been established. The DNA bank will continue to grow and is very likely to serve as a valuable resource for NIOSH and other investigators pursuing investigations of susceptibility genes for OA.

2) **Intermediate outcomes** (how findings,results or recommendations have been used by others to influence practices, legislation, safety management program, training, etc.)

This remains to be determined as this work is still ongoing. We will identify the most relevant genetic markers in the next phase of our studies which will enable use of genetic panels to identify high risk workers. Such workers could be targeted for more frequent worker surveillance compared to low risk workers.

3) **End outcomes** (how findings have contributed to documented reductions in work-related morbidity, mortality, and/or exposure)

The desired end outcomes resulting from use of functional genetic markers for risk assessment and worker surveillance would be: 1) identification of workers early in development of DA so that individuals could be removed early from further harmful exposure to isocyanates; and 2) reduction of new cases of DA.

Section 2: Scientific Report

Background

Diisocyanate chemicals are leading causes of occupational asthma (OA) [1], and estimated to cause OA in 5-15% of chronically exposed workers. The most commonly used in industry are: the aliphatic agent 1,6 hexamethylene diisocyanate (HDI) used principally as a hardener in spray paints, 4,4-diphenylmethane diisocyanate (MDI), and toluene diisocyanate (TDI). Aromatic agents are widely used in polymerization reactions for manufacturing surface coatings, varnishes, paints, urethane foams, insulation, adhesives, binders and sealants etc. Pre-polymerized forms of diisocyanates are more common in commercial products than monomeric moieties [2]. For example, two-component paints are made of nonvolatile pre polymers of HDI (30% to 60%) with only trace amounts of monomer [3]. In a recent review of a surveillance program in the UK, spray painters were at the highest risk of developing occupational asthma compared to other jobs. Isocyanates were the most common causative agents identified; accounting for 17.3% out of the total 1097 reported cases over a seven year period [4].

Workers who experience persistent diisocyanate exposure following commencement of work-related symptoms are more likely to develop long-term asthma disability compared with affected workers removed from the work environment [5]. Failure to recognize diisocyanate asthma (DA) can result in fatal asthma exacerbations [6]. For this reason, medical surveillance programs aimed at secondary prevention have been instituted [7, 8]. Surveillance programs usually involve questionnaires and periodic measures of lung function. A recent report suggests that, compared with other causes of OA, surveillance programs in diisocyanate exposed workers were associated with better long-term clinical outcomes for workers with DA (i.e., 73% cleared or improved versus 56%

for other causes of OA). A better outcome was related to early diagnosis ($P < 0.05$), and early elimination of diisocyanate exposure [9]. We conducted a 3-year intensive surveillance program in 243 assembly workers exposed to MDI, who were surveyed by questionnaire and spirometry. Three workers identified with early onset OA, experienced complete resolution of asthma within 1 year after prompt removal from exposure [10]. However, such programs are disruptive and entail considerable human and financial resources. For these reasons, there is an unmet need for screening methods that can reliably identify those workers that are particularly susceptible to develop OA caused by diisocyanates and other reactive chemicals.

Specific Aims

Despite improved technological advances and industrial hygiene efforts to reduce worker exposure to diisocyanate chemicals (e.g. robotics, with less volatile or reactive diisocyanates), new cases of DA are not entirely preventable and continue to occur [4,10]. It is widely accepted that early diagnosis of OA leads to favorable clinical outcomes (i.e., less risk of chronic and severe asthma) if affected workers are promptly recognized and removed from harmful exposure a causative agent [9]. Strategies that can identify OA due to diisocyanates and other chemicals agents at its earliest stages may reduce the probability of persistent asthma [9]. For this reason, medical surveillance programs have been implemented in industry with the aim of identifying new cases of DA early. These programs result in better clinical outcomes if cases are recognized early and affected workers promptly removed [9,10]. However, beyond early case detection, it would be ideal to identify the most susceptible workers at a pre-clinical stage. In order to address an unmet need, we investigated susceptibility genotypes associated with OA caused by diisocyanates by expanding our ongoing international genetic case control study of OA. We have already identified cytokine gene markers that are expressed at significantly high frequencies in workers with confirmed HDI induced OA [11]. Our second objective was to determine if aforementioned genotypes associated with HDI asthma (i.e., "high risk genotypes") are associated with airway inflammatory responses among asymptomatic workers regularly exposed to HDI. Ultimately, discovery of genetic markers and biomarkers associated with expression of OA can be incorporated into medical surveillance programs and facilitate earlier case identification.

The following three Specific Aims were intended to test the hypothesis that high-risk genotypes will define susceptibility to OA caused by reactive chemicals and predict airway inflammatory responses associated with HDI exposure.

<u>Specific Aim #1</u> ***Expand ongoing case-control studies in workers referred for evaluation of OA due to reactive chemicals to include associations of an expanded panel of genetic polymorphisms*****.**

<u>Cytokine gene variants and DA:</u> In 2006, we reported that DA confirmed by specific inhalation challenge (SIC) testing was significantly associated with cytokine genotype combinations of interleukin 4 receptor alpha (IL4RA), interleukin 13 (IL13) and CD14 single nucleotide polymorphisms (SNPs), but exclusively in HDI exposed workers [11]. In 2011, we confirmed the aforementioned genotype associations in an expanded group of workers with confirmed DA when compared to diisocyanate exposed workers without DA. A total of 368 diisocyanate-exposed workers were recruited by clinical investigators at four occupational disease clinics (Hôpital du Sacré-Coeur de Montréal, Montreal, Canada; Hôpital Laval, Sainte-Foy, Canada; University Health Network, Toronto, Canada; and Fundacion Jimenez Diaz, Madrid, Spain) and included: 103 diagnosed with DA (DA+) based on a positive SIC test; 115 symptomatic workers with negative SIC tests (DA-); and 150 HDI-exposed asymptomatic control spray paint workers. Of 103 workers with DA, 50, 22, and 31 were exposed to HDI, MDI, and TDI, respectively. Of the 115 DA− symptomatic workers, 91, 18, and 6 were exposed to HDI, MDI, and TDI, respectively, while all asymptomatic controls were exposed to HDI.

No significant associations were identified (by the X^2 test) between DA and individual alleles or genotypes of the candidate SNPs for IL-4RA, IL-13, or CD14. However, logistic regression analysis revealed significant interactions of diisocyanate exposure (HDI vs. MDI, TDI) with specific genotype combinations (i.e., IL4RA II + IL13RR; IL4RA II + CD14 CT; and IL4RA II + IL13 RR + CD14 CT) for distinguishing confirmed DA status (DA+) in comparison to SIC negative workers (DA−), after adjusting for significantly associated demographic variables

(Table 1). As shown in Table 2 below, when comparing HDI-exposed DA+ workers (n = 50) with asymptomatic HDI-exposed workers (n = 150), the association between DA and the IL-4RA II 1 CD14 CT and IL-4RA II 1 IL-13 RR1 CD14 CT genotype combinations approached statistical significance (P < .10) after adjustment for age at diagnosis and smoking status.

Table 1. Logistic regression analyses of associations of genotype and genotype combinations and exposure (HDI, MDI, TDI) with DA+ clinical outcome versus DA- (comparator).

Genotype or genotype combinations of IL4RA (I50V), IL13 (R110Q), and CD14 (C159T) SNPs	**P-values**			**OR (95% CI) for genotype among diisocyanate-exposed workers**
	Genotype or genotype combination main effect	**Exposure main effect, HDI vs. MDI, TDI**	**Genotype or genotype combination by exposure interaction**	
All workers				
IL4RA II	.810	.0001	.100	1.57 (0.74, 3.32)[a]
IL4RA II and IL13 RR	.578	.005	.051	1.66 (0.66, 4.17)[a]
IL4RA II and CD14 CT	.895	.033	.006	2.98 (1.25, 7.09)[a]
IL4RA II and IL13RR and CD14 CT	.801	.564	.003	3.55 (1.19, 10.55)[b]
HDI exposed workers only				
IL4RA II	.292			1.51 (0.70, 3.28)[a]
IL4RA II and IL13 RR	.340			1.58 (0.62, 4.06)[a]
IL4RA II and CD14 CT	.015			3.08 (1.25, 7.60)[a]
IL4RA II and IL13RR and CD14 CT	.019			3.86 (1.26, 11.98)[c]

OR, odds ratio. * Each row represents a separate logistic model. Genotypes are dichotomized so that IL4RA II compares the II genotype to the IV and VV genotypes. IL13 compares the RR genotype to the RQ and QQ genotypes, and CD14 CT compares the CT genotype to the CC and TT genotype. Combinations of genotypes compare the indicated SNP combination with all other combinations. For example, IL4RA II and IL13 RR compare the combination of IL4RA (I50V) = II and IL13 (R110Q) =RR with all other combinations of IL4RA (I50V) and IL13 (R110Q).

[a]Adjusted for smoking [b]Adjusted for smoking and ethnicity, [c]Adjusted for smoking and gender

Table 2. Logistic regression analyses in HDI- exposed workers of associations of genotype and genotype combinations after adjusting for significant demographic characteristics

Genotype or genotype combination of IL4RA (I50V), IL13 (R110Q), and CD14 (C159T) SNPs	**P-value**	**OR (95% CI) for genotype[a]**
HDI exposed workers only		
IL4RA II	.151	1.68 (0.83, 3.40)
IL4RA II and IL13 RR	.345	1.51 (0.64, 3.54)
IL4RA II and CD14 CT	.087	2.18 (0.89, 5.30)
IL4RA II and IL13RR and CD14 CT	.093	2.65 (0.85, 8.23)

OR, Odds ratio. *Each row represents a separate logistic model. Genotypes are dichotomized and analyzed as explained in Table 1.

[a]Adjusted for age at diagnosis and smoking.

These results in an expanded group of workers confirm our original findings of significant associations of DA with IL4RA (I50V), IL13 (R110Q), and CD14 (C159T) genotype combinations associated with Th2 and innate immunity modified by exposure to HDI [12]. Unique to this report is the finding that 2 genotype combinations remain significantly associated with DA compared with DA-workers, and a similar, but not statistically significant, association was observed when compared with an asymptomatic cohort of HDI-exposed workers. Significant associations between genotype and occupational asthma were found only after adjustment for work-relevant diisocyanate exposure (ie, HDI vs TDI, MDI). The reason for restriction of this finding to the HDI-exposed population is unknown and may be an artifact of the greater numbers of HDI-exposed subjects available for statistical analysis.

Antioxidant gene variants and DA: Using a case-control approach, we evaluated genetic variations in anti-oxidant enzyme genes [glutathione-s-transferases (GSTM1, GSTP1, and GSTT1), microsomal epoxide hydrolase (EPHX1), superoxide dismutase (SOD), and N-acetyltransferase (NAT1, NAT2)] for association with DA. Candidate genes were selected based on their functional role in oxidative stress and inflammation. We genotyped 410 workers exposed to diisocyanates of which 132 were diagnosed with DA, using a 5' nuclease PCR assay. Workers cohorts comprised of three phenotypes including: 1) 132 workers diagnosed with DA (DA+) based on a positive specific inhalation challenge (SIC) test; 2) 130 workers reporting respiratory symptoms at work in whom DA was excluded (DA-) based on a negative SIC; and 3) 148 HDI-exposed asymptomatic worker controls (AWs).

Statistical analysis was conducted by first examining potential associations between each SNP and DA using chi- square tests for single SNP associations. Mantel-Haenszel chi-square statistics and the associated Breslow-Day tests for homogeneity of odds ratios were used to investigate pairs of SNPs associated with diagnosis ($p < 0.05$). Logistic regression analysis was used to examine these pairings with adjustment for the effects of the demographic variables that retained significance when included in the model (ethnicity, smoking, type of diisocyanate exposure and exposure duration). Then, backward selection was used to choose from among the SNPs and their two-way interactions with statistical significance at $p < 0.05$. The rs4880 (SOD2) and rs2740171 (EPHX1) were the only candidate SNPs that were individually significantly associated with the DA diagnosis. The frequency of the SOD2 rs4880 Val105Val genotype was significantly different in DA+ workers (38%) compared to DA- workers ($p=0.009$) and AW controls ($p=0.002$) (21% in both). Distribution of minor allele homozygotes for the SNP rs2740171 was also significantly different among DA+ cases (8.3%) compared to AW controls (4.7%) ($p=0.010$).

Logistic regression models examined significant pairings associated with DA after adjusting for significant confounders. The first model included DA+ and DA- groups and adjusted the results for smoking and specific diisocyanate exposure (Table 3). SOD2 rs4880 SNP Val105Val genotype was associated with a higher risk of DA with an OR of 2.89 (95% CI, 1.49-5.60). GSTP1 rs1695 SNP also showed an association with DA under this model (OR, 6.67; 95% CI, 1.9- 29.99). On the other hand, GSTM1 (del) and EPHX1 rs2854450 variations conferred protection against DA (OR, 0.44; 95% CI, 0.21-0.93 and OR, 0.19, 95% CI, 0.08-048, respectively). Some SNPs conferred risk only in the presence of one another SNP. The co-presence of GSTP1 rs1695- GSTT1 (del) and GSTP1 rs762803- EPHX1 rs2740168 was associated with a lower risk of DA (OR, 0.12; 95% CI, 0.03-0.59 and OR, 026; 95% CI, 0.08-0.86, respectively). Although both the GSTM1 (del) and EPHX1 rs2854450 SNP were individually associated with protection against DA, the presence of both variants conferred an increased risk of disease with an OR of 8.22 (95% CI, 2.30-29.35).

Table 3. Logistic regression model for significant variations, DA+ vs DA- groups

Model term	Estimate, β	S.E.	χ^2	OR (95% CI)	p-value†
Intercept	0.2525	0.3620	0.49		0.4855
SOD2 (rs4880)	0.5310	0.1687	9.90	**2.89 (1.49, 5.60)**	**0.0017**
GSTP1 (rs1695)	0.9491	0.3833	6.13	**6.67 (1.49, 29.99)**	**0.0133**
GSTT1 (del)	0.0600	0.2724	0.05	1.13 (0.39, 3.28)	0.8256
GSTP1 (rs762803)	0.1414	0.2766	0.26	1.33 (0.45, 3.92)	0.6093
GSTM1 (del)	-0.4058	0.1871	4.71	**0.44 (0.21, 0.93)**	**0.0301**
EPHX1 (rs2854450)	-0.8370	0.2390	12.27	**0.19 (0.08, 0.48)**	**0.0005**
EPHX1 (rs2740168)	0.2637	0.2440	1.17	1.69 (0.65, 4.41)	0.2798
GSTP1(rs1695)*GSTT1(del)	-1.0523	0.4017	6.86	**0.12 (0.03, 0.59)**	**0.0088**
GSTM1 (del)*EPHX1(rs2854450)	1.0530	0.3248	10.51	**8.22 (2.30, 29.35)**	**0.0012**
GSTP1 (rs762803)*EPHX1(rs2740168)	-0.6758	0.3054	4.90	**0.26 (0.08, 0.86)**	**0.0269**

†Adjusted for smoking (current vs never) and diisocyanate exposure

The second logistic regression model included DA+ (n= 60) and AW (n=147) groups and adjusted the results for age, ethnicity and exposure months (Table 4). Only HDI-induced DA+ cases were taken into consideration since controls were exposed only to HDI. While the GSTT1 (del) and EPHX1 rs2740168 and EPHX1 rs2854450 minor alleles were significantly associated with protection against DA (OR, 0.07; 95% CI, 0.01-0.30; OR, 0.16; 95% CI, 0.03-0.80; OR, 0.29; 95% CI, 0.11-0.80, respectively), carriage of the minor allele for EPHX1 rs2740171 conferred an increased risk for DA with an OR of 9.53 (95% CI, 3.24-28.01). The co-presence of GSTT1 (del) and EPHX1 rs2740168 was also associated with an increased risk of DA (OR, 23.51; 95% CI, 2.87-192.72) [13].

Table 4. Logistic regression model for significant variations, DA+ (n=60) vs AW controls (n=147)

Model term	Estimate, β	S.E.	χ^2	OR (95% CI)	p-value†
Intercept	-5.1497	1.0170	25.64		<0.0001
GSTT1 (del)	-1.3692	0.3910	12.26	**0.07 (0.01, 0.30)**	**0.0005**
EPHX1 (rs2740168)	-0.9325	0.4197	4.94	**0.16 (0.03, 0.80)**	**0.0263**
EPHX1 (rs2854450)	-0.6299	0.2578	5.78	**0.29 (0.11, 0.80)**	**0.0162**
EPHX1 (rs2740171)	1.1270	0.2752	16.77	**9.53 (3.24, 28.01)**	**<0.0001**
GSTT1 (del)*EPHX1 (rs2740168)	1.5788	0.5367	8.66	**23.51 (2.87,192.72)**	**0.0033**

†Adjusted for age, ethnicity and exposure months

Taken together, this case-control study reports that the SOD2 rs4880, EPHX1 2740171 and GSTP1 rs1695 variants are significantly associated with DA supporting the hypothesis that genetic variability within antioxidant defense systems contributes to the pathogenesis of this disease. After adjustment for confounding variables, variants of GSTM1, GSTT1, GSTP1, EPHX1 and GSTM3 genes also showed significant positive or negative associations with DA. This may suggest that the effect of these variations can be detected after the variability of the outcome is reduced by adjusting for the other associated factors and in the context of gene-gene, gene-exposure interactions. Our results support the involvement of these variations in asthma, particularly in asthma phenotypes driven by oxidant stress-dependent pulmonary inflammation.

N-acetyl transferase SNPs which have also been genotyped in the aforementioned antioxidant gene studies are currently being analyzed as well.

MHC gene variants and DA: We evaluated genetic variations in major histocompatibility region (MHC) genes for association with DA. Genotyping was performed using the Illumina GoldenGate MHC panels. The study population consisted of 140 workers exposed to diisocyanates of which 73 were diagnosed with DA based on a positive SIC and 67 AWs exposed to HDI. After adjusting for potential confounders and for multiple comparisons by the Bonferroni method, SNPs mapping to HLA-E, HLA-B, HLA-DOA, HLA-DQA2, and HLA-DPB1 genes showed associations with altered risk of developing DA under dominant and recessive genetic models (Table 5). The HLA-E rs1573294 and HLA-DPB1 rs928976 SNPs were associated with an increased risk of DA under dominant (OR, 6.27; 95% CI, 2.37-16.6; OR; 2.79; 95% CI, 0.99-7.81, respectively) and recessive genetic models (OR, 6.27; 95% CI, 1.63-24.13; OR, 10.10; 95% CI, 3.16-32.33, respectively). The HLA-B rs1811197, HLA-DOA rs3128935 and HLA-DQA2 rs7773955 SNPs conferred an increased risk of DA in a dominant model (OR, 7.64; 95% CI, 2.25-26.00; OR, 19.69, 95% CI, 2.89-135.25, OR, 8.43; 95% CI, 3.03-23.48, respectively) [14].

Table 5. Association of HLA SNPs with DA under different genetic models

Gene	dbSNP ID	Dominant Model Unadjusted OR (95% CI)	P value	Dominant Model Adjusted† OR (95% CI)	P value	Recessive Model Unadjusted OR (95% CI)	P value	Recessive Model Adjusted† OR (95% CI)	P value
HLA-E	rs1573294	5.55 (2.68, 11.51)	<0.0001	6.27 (2.37, 16.61)	0.0002	9.80 (3.21, 29.89)	<0.0001	6.27 (1.63, 24.13)	0.0076
HLA-B	rs1811197	10.23 (3.68, 28.40)	<0.0001	7.64 (2.25, 26.00)	0.0001	8.74* (0.46, 165.5)	0.0519	**	
HLA-DOA	rs3128935	17.98 (4.07, 79.48)	<0.0001	19.69 (2.89,135.25)	0.0024	15.22* (0.85, 271.9)	0.0093	**	
HLA-DQA2	rs7773955	9.20 (4.12, 20.57)	<0.0001	8.43 (3.03, 23.48)	<.0001	35.76* (2.09, 610.9)	<0.0001	**	
HLA-DPB1	rs928976	3.63 (1.71, 7.72)	0.0009	2.79 (0.99, 7.81)	0.0511	13.00 (5.22, 32.39)	<0.0001	10.10 (3.16, 32.33)	<0.0001

DA+, symptomatic workers diagnosed with diisocyanate asthma; AW, asymptomatic workers; OR, odds ratio; CI, confidence interval †Logistic regression models were adjusted for age, gender, atopy, height, exposure duration, and smoking * Logit estimators used to adjust for cells with 0 count, ** Models failed to converge due to cells with 0 counts

Taken together, the present study showed novel associations between SNPs in MHC Class I (HLA-E, HLA-B) and Class II (HLA-DOA, HLA-DQA2 and HLA-DBP1) genes and DA.

The present results are consistent with the hypothesis that an immunological mechanism is involved in DA and that genetic variations within HLA genes play a major role in DA risk. Identification of significant polymorphisms and their allelic variations within the MHC is potentiallly important as the structural diversity of the MHC alleles influence peptide binding and control disease susceptibility. We plan further studies to validate the results reported herein and identify causative alleles behind these associations using high resolution mapping.

CTNNA3 SNPs and DA: We also conducted a candidate gene association study to replicate the findings from the only reported a Korean GWAS study of DA demonstrating an association between two closely linked CTNNA3 gene SNPs and DA [15]. Genotyping was performed on DNA using a 5' nuclease PCR assay collected from 410 diisocyanate exposed and predominantly Canadian workers including: 132 workers with DA confirmed by a specific inhalation challenge (DA+); 131 symptomatic workers in whom DA was excluded by a negative challenge (DA-); and 147 HDI-exposed asymptomatic workers (AWs). As in the Korean study, highly linked CTNNA3 rs7088181 and rs10762058 SNPs were significantly associated with DA+ when compared to AWs (Tables 6 and 7) but not in comparison to DA- workers ($p \leq 0.05$). After adjusting for potentially confounding variables of age, smoking status and duration of exposure, minor allele homozygotes of rs7088181 and rs10762058 SNPs were at increased risk for DA compared with AWs [OR= 9.05 (95% CI:1.69, 48.54) and OR = 6.82 (95% CI:1.65, 28.24), respectively] [16].

In summary, we have replicated results from a GWAS study demonstrating an association between two linked SNPs of the CTNNA3 gene and DA. Our findings suggest that genetically determined reduced

expression of alpha-T-catenin might influence cellular adherence in the airways and could play a role in the pathogenesis of DA. These results lend further support to the clinical relevance of these genotypes in predicting susceptibility to DA.

Table 6. Logistic regression model for CTNNA3 rs7088181 (DA+ vs AW comparator group)

Effect (recessive model)	**Odds ratio (95% confidence intervals)**	**P value[£]**
CTNNA3 rs7088181 (1.1+1.2) vs 2.2*	9.05 (1.69, 48.54)	0.01
Age (yrs)	1.10 (1.06 1.14)	<0.0001
Smoking (current vs. never)	0.31 (0.15, 0.64)	0.0017
Exposure duration (months)	1.01 (1.00, 1.01)	0.0019

*Genotypes, 1.1: homozygous for the major allele; 2.2: homozygous for the minor allele; 1.2 heterozygous
[£] The results were adjusted for age, smoking, and exposure duration

Table 7. Logistic regression model for CTNNA3 rs10762058, DA+ versus AW comparator group

Effect (recessive model)	**Odds ratio (95% confidence intervals)**	**P value**
CTNNA3 rs10762058 (1.1+1.2) vs 2.2*	6.82 (1.65, 28.24)	0.008
Age (yrs)	1.10 (1.06, 1.14)	<0.0001
Smoking (current vs. never)	0.32 (0.15, 0.67)	0.0024
Exposure duration (months)	1.01 (1.00, 1.01)	0.0020

*Genotypes, 1.1: homozygous for the major allele; 2.2: homozygous for the minor allele; 1.2 heterozygous
[£] The results were adjusted for age, smoking, and exposure duration

<u>Specific Aim #2:</u> ***Evaluate associations between high-risk genotypes in asymptomatic HDI-exposed workers, airway inflammatory responses and airway responsiveness during workplace exposure to HDI.***

As proposed, 150 asymptomatic HDI exposed spray paint workers have been recruited in by our collaborator in Montreal, Dr. Denyse Gautrin. Dr. Gautrin enrolled 150 HDI workers which we have genotyped for high risk genotypes or genotype combinations of IL4Rα, IL-13 and CD14 SNPs (see Table 8 below) that in our early studies were found to predict DA caused by HDI. We have subsequently identified 15 asymptomatic workers with high risk genotype profiles (defined by IL4 receptor-alpha genotype ILRa(II) alone or combined with IL-13 (R110Q) RR and/or CD14 (C159T) CT genotypes) and 15 with low risk genotype profiles (from their Montreal, Canada cohort of 150 asymptomatic HDI exposed workers) and completed prospective serial workplace monitoring for inflammatory markers in induced sputum (i.e., sputum eosinophils and LTB4, IL-8 and MMP9 levels in sputum supernatants) by evaluating HDI exposed spray painting workers after 2 weeks away from work (baseline) and again after 2 weeks of HDI exposure at work. Serial measurements of methacholine PC20 were conducted along with sputum markers. Sputum processing and counting of eosinophils were performed in the laboratory of our co-investigator, Dr. Catherine Lemiere. The purpose of this study was to determine if high risk genetic markers predicted susceptibility to early airways inflammatory changes associated with natural isocyanate exposure in the workplace.

Preliminary results among 30 workers studied were compared between high risk and low risk HDI exposed workers. No significant differences between group changes from baseline measurement have been identified for:

spirometry measures (FEV1, FVC); methacholine PC20; sputum inflammatory cells expressed as percent of total inflammatory cells including eosinophils, neutrophils, lymphocytes, macrophages; and measured IL-5, IL-8 cytokine levels in sputum supernatants. A trend was detected suggesting MMP9 concentrations in induced sputum increased in high risk genotype workers versus low risk workers in association with work exposure to HDI (mean change -7943 vs. +83,303 pg/ml; p=0.076). In contrast an opposite trend was observed for LTB4 showing a decline in high risk versus low risk genotype groups associated with HDI exposure (p=0.086). The latter trends were evident when data was analyzed by both parametric and non-parametric statistics. Inability to reach significant differences could be attributed to the small numbers of available sputum supernatant samples (n=10 and n=11 for low and high groups, respectively).

Specific aim 3: ***Determine if airway inflammatory responses and/or changes in airway responsiveness observed at work in asymptomatic spray painters (specific aim 2) are elicited by HDI exposure.***

This specific aim entailed performance of controlled SIC tests with HDI in the aforementioned subjects at a specialized laboratory located at Hospital Sacré Coeur (Montreal, CA) under supervision of foreign collaborators, Drs. Cartier and Lemiere. Because results of Aim 2 were not informative, the investigators cancelled proposed SIC in aim 3, as risks posed by laboratory exposure to the isocyanates could not be ethically justified by low potential for new knowledge gained from the proposed research.

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Inclusion Enrollment Report

This report format should NOT be used for data collection from study participants.

Study Title: Genetic Susceptibility for Occupational Asthma

Total Enrollment: 634 **Protocol Number:** Exemption 4

Grant Number: R01 OH008795

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race

Ethnic Category	Sex/Gender			
	Females	Males	Unknown or Not Reported	Total
Hispanic or Latino	2	7	0	9 **
Not Hispanic or Latino	117	503	0	620
Unknown (individuals not reporting ethnicity)	2	3	0	5
Ethnic Category: Total of All Subjects*	121	513	0	634 *
Racial Categories				
American Indian/Alaska Native	0	0	0	0
Asian	0	3	0	3
Native Hawaiian or Other Pacific Islander	0	1	0	1
Black or African American	0	3	0	3
White	119	504	0	623
More Than One Race	0	0	0	0
Unknown or Not Reported	2	2	0	4
Racial Categories: Total of All Subjects*	121	513	0	634 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)

Racial Categories	Females	Males	Unknown or Not Reported	Total
American Indian or Alaska Native	0	0	0	0
Asian	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0
Black or African American	0	0	0	0
White	2	7	0	9
More Than One Race	0	0	0	0
Unknown or Not Reported	0	0	0	0
Racial Categories: Total of Hispanics or Latinos**				9 **

* These totals must agree.
** These totals must agree.

Progress Report: Publication List

1. **Bernstein D,** Kissling, GE, Khurana Hershey G, Yucesoy B, Johnson VJ, Cartier A, Gautrin, D, Sastre J, Boulet LP, Malo JL, Quirce S, Tarlo S, Langmeyer S, Luster MI , Lummus ZL: [2011] Hexamethylene Diisocyanate Asthma is Associated with Genetic Polymorphisms of CD14, IL-13, and IL-4RA, Journal of Allergy and Clinical Immunology, 128:418-420. **PMID: 21489615 (Specific Aim 1)**
2. Yucesoy B, Johnson VJ, Lummus ZL, Kissling G, Fluharty K, Gautrin D, Malo JL, Cartier A, Boulet LP, Sastre J, Quirce S, Germolec D, Tarlo SM, Cruz MJ, Munoz X, Luster M, **Bernstein DI**: [2012] Genetic variants in antioxidant genes are associated with diisocyanate-induced asthma, Toxicological Sciences, 129:166-173. **PMID:22610343 (Specific Aim 1)**
3. **Bernstein DI**, Kashon M, Lummus ZL, Johnson VJ, Fluharty K, Gautrin D, Malo JL, Cartier A, Boulet LP, Sastre J, Quirce S, Germolec D, Tarlo SM, Cruz MJ, Munoz X, Luster MI, Yucesoy B: [2013] CTNNA3 (α-catenin) gene variants are associated with diisocyanate asthma, a replication study in a Caucasian worker population. Toxicological Sciences, 131:242-6. **PMID:22977168 (Specific Aim 1)**
4. Yucesoy B, Johnson VJ, Lummus ZL, Kashon ML, Frye B, Wang W, Marepalli R, Thompson HB, Gautrin D, Malo JL, Cartier A, Boulet LP, Sastre J, Quirce S, Tarlo SM, Germolec DR, Luster MI, **Bernstein DI**: [2014] Genetic variants in the MHC class I and class II genes are associated with diisocyanate-induced asthma, Journal of Occupational and Environmental Medicine, 56:382-387. **PMID: 24709764 (Specific Aim 1)**
5. Lummus ZL, Wisnewski AV, **Bernstein DI:** [2011] Pathogenesis and disease mechanisms of occupational asthma. Immunology and Allergy Clinics of North America, 31:699–716. **PMID:21978852 (Review article)**
6. Malo J-L, L'Archeveˆque J, Lummus Z, **Bernstein DI:** [2006] Changes in specific IgE and IgG and monocyte chemoattractant protein-1 in workers with occupational asthma caused by diisocyanates and removed from exposure. Journal of Allergy and Clinical Immunology, 118:530-533. **PMID:16890787 (Background: innate and humoral immune markers of DA)**
7. Campo P, Wisnewski AV, Lummus Z, Cartier A, Malo J-L, Boulet LP, **Bernstein D:** [2007] Diisocyanate conjugate and immunoassay characteristics influence detection of specific antibodies in HDI-exposed workers. Clinical & Experimental Allergy, 37:1095-1102. **PMID:17581205 (Background: antibody responses in DA)**
8. **Bernstein DI**. Genetics of occupational asthma: [2011] Current Opinion in Allergy and Clinical Immunology, 11:86-89. **PMID:21325943 (Review article)**