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Sex-Based Analysis of Potential Inflammation-Related Protein Biomarkers in the Aqueous Humor of Patients With Diabetes Mellitus

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Purpose: To investigate whether men have higher inflammatory protein biomarker concentrations in their aqueous humor (AH) compared with women in groups of patients with varying levels of diabetic disease.

Methods: This cross-sectional study included AH specimens from 59 adult patients comprised of three groups: no diabetes mellitus (DM), DM without diabetic retinopathy (DR), and DM with proliferative diabetic retinopathy (PDR). Protein biomarker concentration values were quantified using a commercial proximity extension assay-based technique.

Results: Intersex comparisons of concentration values for each protein biomarker revealed no discoveries in patients with no DM or with PDR. In contrast, 24 discoveries were detected in patients with DM without DR. The mean concentration value for all 24 protein biomarkers was higher in men compared with women. Of these 24 proteins, 12 demonstrated a significant association with sex on multivariate linear regression analysis. The β coefficient results demonstrated a positive association between male sex and concentration value for all 12 of these proteins.

Conclusions: Higher AH concentration levels of several potential biomarkers, including chemokines, proteases, proteins involved in programmed cell death, and a T-cell surface protein, were detected in men with DM with no DR. These findings suggest that men may have a more inflammatory disease phenotype compared with women in this group of patients.

Translational Relevance: The findings of this study help explain differences in epidemiologic patterns of diabetic retinopathy development between men and women.

Introduction

Biological sex plays an important role in ophthalmic anatomy, physiology, and disease.[1,2] For example, corneal thickness and intraocular pressure measurements in women vary throughout the menstrual cycle and pregnancy. In addition, results from large population-based studies suggest that women are at higher risk for age-related macular degeneration, normal-tension glaucoma, and angle-closure glaucoma.[3] In the context of diabetes mellitus (DM), men appear to be at higher risk for microvascular complications of the disease.[4,5] Indeed, major epidemiologic studies in Western populations have reported a higher incidence of diabetic retinopathy (DR) in men and a lower of risk of DR progression in women.[6–8] The mechanism underlying these sex-based differences is not well understood.

DR is a complex microvascular disease involving multiple aberrant processes. Inflammation is thought to be an important component in its pathogenesis.[9,10] Over the last several years, a number of potential DR protein biomarkers involved in retinal inflammatory pathways have been identified in various biofluids.[11] Perturbations in the levels of these cytokines may play a role in the clinically observed discrepancies in DR between men and women. Specifically, we

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hypothesize that men with DM may have higher levels of inflammatory protein biomarkers in their intraocular fluid. In this study, we sought to test this hypothesis by comparing aqueous humor (AH) protein biomarker concentrations between men and women in groups of patients with varying levels of diabetic disease.

Materials and Methods

This study was approved by the Institutional Review Board at the University of California, San Francisco, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all involved patients.

Clinical Data

This study was conducted at the University of California, San Francisco and the Zuckerberg San Francisco General Hospital and Trauma Center. In total, 60 adult patients (age 18 years or older) were recruited to construct three age- and sex-balanced (1:1) study groups comprised of 20 patients per group: patients with no history of DM, patients with a history of DM with no DR, and patients with a history of DM with proliferative DR (PDR). Patients with nonproliferative DR were not considered for inclusion because of limitations in specimen availability and the relatively wide spectrum of ophthalmic disease that is present within this cohort. Patients with known inflammatory ocular comorbidities were also excluded. All patients underwent aqueous humor specimen collection at the time of a medically indicated procedure between May 2018 and October 2019. Such interventions included cataract extraction and intraocular lens implantation (CEIOL), intravitreal injection (IVI), and pars plana vitrectomy (PPV).

The electronic health record for each patient was reviewed to retrieve demographic and clinical information. The latter included relevant systemic comorbidities, lens status at the time of AH specimen collection, ocular comorbidities, and details related to diabetic disease, retinopathy status, and treatment history.

Proteomic Data

AH specimens were collected by anterior chamber paracentesis with the use of a 30-gauge needle on a 1 mL tuberculin syringe prior to initiation of the concomitant procedure. Specimens were stored in a freezer at 80° C until they were sent to Olink Proteomics (Uppsala, Sweden) for analysis. Potential protein biomarkers from the Olink Immuno-Oncology panel (v.3111) were evaluated using a Proximity Extension Assay (PEA) technique, which has been described in detail elsewhere.[12] This particular panel was chosen because of its unique inclusion of a variety of both inflammatory cytokines and growth factors. A full list of potential protein biomarkers included in this panel (92 in total) and associated assay validation data (e.g., limit of detection [LOD], lower and upper limits of quantification, within- and between-run precision coefficient of variation) can be found on the manufacturer's website (https://www.olink.com). The final protein concentration output from these assays is reported in Normalized Protein eXpression (NPX) values. The NPX is an arbitrary unit on a log base 2 scale wherein higher NPX values correlate with higher protein concentrations. For example, a 1-point difference in an NPX value is equivalent to a twofold change in protein concentration.

Protein expression data results from 60 AH specimens from 60 patients were obtained. One specimen from a patient with DM with PDR failed the manufacturer's quality control test and was excluded. Thirty-two potential protein biomarkers were found to have a high frequency (50% or greater) of NPX values below the LOD and were not considered for data analysis in accordance with the manufacturer's recommendations. These included the following: monocyte chemotactic protein 3, CD40 ligand, interleukin-1 alpha, natural killer cell receptor, pro-epidermal growth factor, adhesion G-protein–coupled receptor G1, cytotoxic and regulatory T-cell molecule, fibroblast growth factor 2, mucin-16, endothelial nitric oxide synthase, interleukin-2, granzyme H, killer cell immunoglobulin-like receptor 3DL1, tumor necrosis factor ligand superfamily member 14, programmed cell death protein 1, fas antigen ligand, T-cell-specific surface glycoprotein, interleukin-5, CD70 antigen, interleukin-10, arginase-1, natural cytotoxicity triggering receptor 1, stromal cell-derived factor 1, interferon gamma, lysosome-associated membrane glycoprotein 3, programmed cell death 1 ligand 2, interleukin-4, lymphocyte activation gene 3 protein, interleukin-12 receptor subunit beta-1, interleukin-13, tumor necrosis factor, and natural killer cells antigen CD94. As a result, the final data set described 60 potential protein biomarkers in 59 aqueous humor specimens from 59 patients. All remaining potential protein biomarker concentrations with NPX values below the LOD were included in the final analysis; they were neither replaced with a specific value nor excluded to increase statistical power and reduce distribution skew.

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Table 1. Clinical Data for Each Study Group Stratified by Sex

	No DM			DM With No DR			DM With PDR		
Variable	Women	Men	*P* Value*	Women	Men	*P* Value	Women†	Men	*P* Value
Age (years)	69.5 ± 6.2	71.9 ± 9.7	NS	66.0 ± 5.9	65.9 ± 7.1	NS	53.9 ± 9.3	58.3 ± 6.9	NS
Hypertension	7	7	NS	7	9	NS	9	7	NS
Hyperlipidemia	4	4	NS	10	9	NS	7	6	NS
Obesity	1	0	NS	7	5	NS	4	2	NS
Current Smoker	1	2	NS	0	4	0.09	0	1	NS
Type 2 DM	—	—	—	10	9	NS	8	10	NS
Insulin Use	—	—	—	2	1	NS	5	4	NS
HbA1c‡ (%)	—	—	—	8.2 ± 2.5	7.4 ± 1.5	NS	10.6 ± 3.0	7.6 ± 1.7§	0.02
Phakic	10	10	NS	10	9	NS	8	9	NS
Ocular Comorbidities	PXE (1) GS (1)	—	—	LTG (1) GS (3) PACG (1)	GS (1)	—	GS (1)	POAG (1)	—

*All *P* values > 0.10 are reported as NS (not significant).

†All sex-based sub-groups were of equal size (n = 10) with the exception of women in group 3 (n = 9).

‡The most recent value obtained prior to aqueous humor specimen collection was used in this study.

§One man with DM with PDR did not have a documented HbA1c.

HbA1c, hemoglobin A1c; PRP, panretinal photocoagulation; PXE, pseudoexfoliation; GS, glaucoma suspect; LTG, low-tension glaucoma; PACG, primary angle closure glaucoma; POAG, primary open-angle glaucoma.

Statistical Analysis

Sample size calculations to determine appropriate group sizes were unable to be performed because of a lack of previously published data on the studied topic. Descriptive and inferential statistics were performed with Stata 16 (StataCorp, College Station, TX, USA) and Prism 8 (GraphPad Software, San Diego, CA, USA). Continuous variables are presented as a mean ± standard deviation and were compared with a Student's *t*-test. Categorical variables are presented as raw data and were compared with a Fisher's exact test because of low values.

Intersex comparisons of mean NPX values for each protein were performed within each study group without assuming a constant variance. A false discovery rate approach using the two-stage step-up method of Benjamini, Krieger, and Yekutieli was used to address the issue of multiplicity.[13] The q-value was set to 10% given the exploratory intent of the study. If a discovery was detected, multivariate linear regression was performed for the implicated protein biomarker with the NPX value as the dependent variable and sex as the independent variable in addition to adjusting for the following covariates: age, systemic hypertension, hyperlipidemia, obesity (body mass index ≥ 30), current smoker status, insulin use, and most recent hemoglobin A1c. $P < 0.05$ was considered statistically significant.

Results

All specimens from patients with no DM or DM with no DR were collected at the time of CEIOL with the exception of one patient with DM without DR whose collection occurred during a PPV performed for a typical macular pucker. Specimens from patients with DM with PDR were collected at the time of PPV (n = 14), CEIOL (n = 3), or IVI (n = 3). Out of all patients with DM with PDR and with a history of IVI, only 2 patients (1 man and 1 woman) had received their last bevacizumab or aflibercept treatment within 30 or 60 days of specimen collection, respectively.

Relevant clinical data for all patient groups stratified by sex are summarized in Table 1. No significant sex-based differences were present in patients with no DM or DM with no DR. In contrast, women with DM with PDR had a significantly higher hemoglobin A1c level ($P = 0.019$) and a trend towards a higher frequency of prior panretinal photocoagulation (6 [67%] versus 2 [20%], $P = 0.07$) when compared to men. However, there was no significant difference in the frequency of prior IVI (6 [67%] versus 6 [60%], $P = 1.0$).

Results of intersex comparisons of NPX values for each potential protein biomarker are presented in the form of study group-specific volcano plots in Figure 1. No discoveries were detected in patients with no DM (Fig. 1A) or with DM with PDR (Fig. 1C). The results from patients with DM with PDR did not change after

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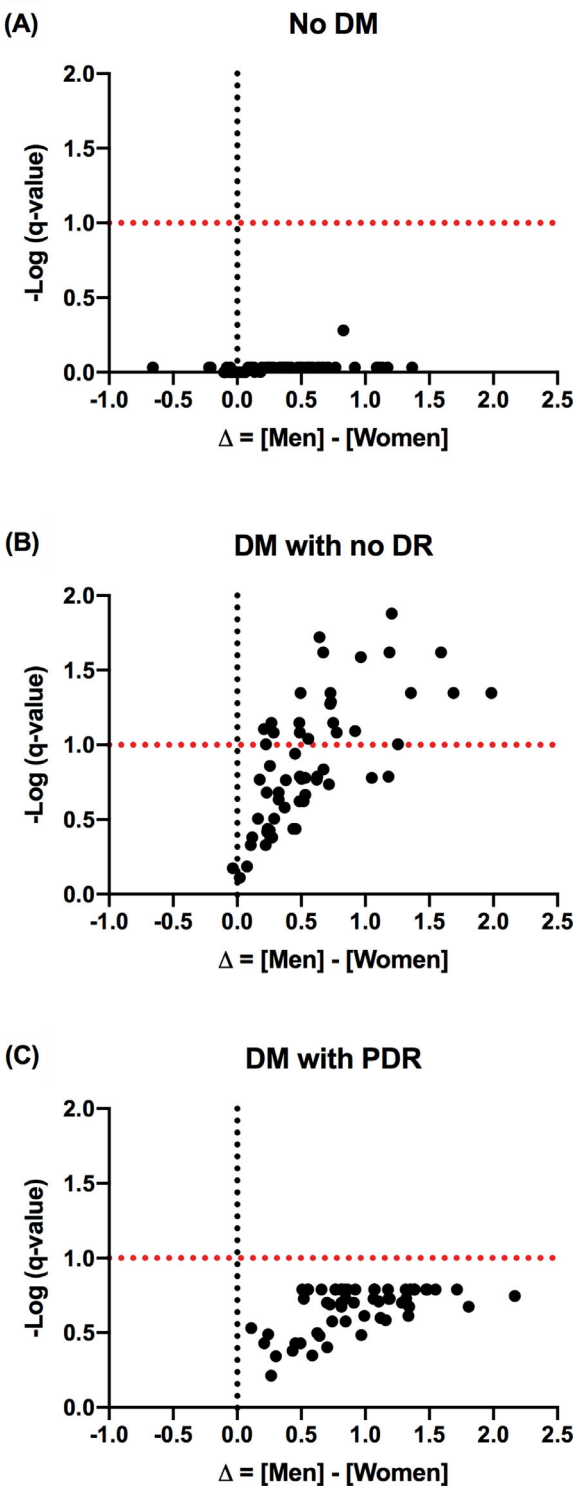

Figure 1. Volcano plots showing negative logarithm of statistical significance (q-value) versus intersex mean protein biomarker concentration differences in patients with no DM (**A**), DM without DR (**B**), and DM with PDR (**C**). No significant differences were detected in patients with no DM or DM with PDR; however, 24 significant differences were detected in patients with DM with no DR.

the exclusion of one patient with type 1 DM or two patients who had recently received an IVI.

In contrast, 24 discoveries were detected in patients with DM with no DR (Fig. 1B, Table 2). These results did not change after the exclusion of one patient with type 1 DM. The mean NPX value for all 24 potential protein biomarkers was higher in men compared with women. Of these 24 potential protein biomarkers, 12 demonstrated a significant association with sex on multivariate linear regression analysis (Table 3). The β coefficient results demonstrated a positive association between male sex and NPX value for all 12 potential protein biomarkers that were assessed. In addition, the β coefficient for sex was several-fold higher than the corresponding β coefficient for hemoglobin A1c for each potential protein biomarker.

Discussion

In diabetes, altered biochemical pathways lead to glial cell activation with subsequent inflammation and secondary vascular dysfunction.[10] In this setting, elevated levels of numerous inflammatory proteins have been found in the serum, vitreous humor (VH), AH, and tears of affected patients.[10,11,14] Significant attention has been paid to the study of these potential biomarkers in patients with DR in recent years.[11] Although the VH may be the preferred ocular biofluid for study of such proteins because of its proximity to the retina, specimen collection requires a relatively invasive procedure with potential sight-threatening complications. In contrast, AH sampling may pose fewer risks to the patient. In addition, the potential biomarker profiles of the AH and VH are highly correlated because of direct diffusion of retinal proteins through the VH-AH barrier and communication through the cilia-retina circulation.[14–16]

In this cross-sectional study, we used a proteomic approach with a relatively novel PEA technique to analyze the concentration of 60 potential protein biomarkers in AH specimens from three groups of patients. Although patients with DM with no DR demonstrated a large number of significant sex-based differences in potential biomarker concentrations, no such results were found in patients with no DM or in patients with DM with PDR.

The results from patients with DM with PDR may be somewhat unexpected. However, any sex-based differences in potential biomarker concentrations in patients with DM may not persist in patients with PDR because of the severity of their disease and its associated inflammation and vascular leakage. Indeed,

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Table 2. Intersex Comparison of Protein NPX Values in Patients With DM With no DR

Protein Biomarker	[Women]*	[Men]*	Δ (Men – Women)†	Q-Value
C-C motif chemokine 23	0.04	1.25	1.21	0.01
T-cell surface glycoprotein CD8 alpha chain	−0.35	0.29	0.64	0.02
Monocyte chemotactic protein 2	0.39	1.58	1.19	0.02
C-X-C motif chemokine 1	1.34	2.94	1.59	0.02
Granzyme A	0.73	1.40	0.67	0.02
Matrix metalloproteinase-12	0.31	1.27	0.97	0.03
Matrix metalloproteinase-7	3.67	5.65	1.98	0.04
Monocyte chemotactic protein 1	9.21	10.57	1.36	0.04
Monocyte chemotactic protein 4	0.09	0.82	0.72	0.04
C-X-C motif chemokine 10	2.27	3.96	1.69	0.04
Angiopoietin-1	−0.03	0.46	0.49	0.04
Fractalkine	2.31	3.04	0.73	0.05
C-C motif chemokine 17	−0.38	0.35	0.73	0.05
C-X-C motif chemokine 11	−0.39	−0.12	0.27	0.07
Casepase-8	0.16	0.64	0.48	0.07
Interleukin-7	1.90	2.65	0.75	0.07
Interleukin-33	0.04	0.25	0.21	0.08
Hepatocyte growth factor	6.04	6.96	0.92	0.08
Angiopoietin-2	−0.27	0.22	0.49	0.08
Vascular endothelial growth factor receptor 2	2.39	3.16	0.78	0.08
T-cell surface glycoprotein CD5	−0.59	−0.30	0.28	0.08
TNF-related apoptosis-inducing ligand	0.49	1.05	0.56	0.09
Interleukin-8	2.48	3.74	1.26	0.10
Platelet-derived growth factor subunit B	−0.17	0.06	0.22	0.10

TNF, tumor necrosis factor.
*Mean NPX values are presented.
†Delta values may appear to be arithmetically inaccurate due to rounding.

patients with DR have been shown to have higher total AH protein levels compared with normal controls.[15] In addition, the women in this group had significantly worse glycemic control compared with their male counterparts, which may have offset any significant sex-based differences. However, the possibility that no such difference exists in patients with PDR should also be considered because other risk factors, such as systemic comorbidity status, may be more deterministic compared to sex in this cohort.

Analysis of the patients with DM with no DR revealed significant sex-based differences in 24 inflammatory protein biomarkers, all of which were higher in men compared with women. The concentrations of 12 of these potential biomarkers remained positively associated with male sex after multivariate regression. In addition, the impact of male sex was larger than at least a four-point increase in HbA1c on the AH concentration for all 12 of the implicated biomarkers. All 12 proteins play a role in inflammation: seven are chemokines (C-C motif chemokine 23, monocyte chemotactic protein 2, C-X-C motif chemokine 1, monocyte chemotactic protein 4, C-X-C motif chemokine 10, C-C motif chemokine 17, C-X-C motif chemokine 11), two are proteases (matrix metalloproteinase-12, matrix metalloproteinase-7), two are involved in programmed cell death (granzyme A, tumor necrosis factor–related apoptosis-inducing ligand), and one is a T-cell surface protein (T-cell surface glycoprotein CD8 alpha chain). These results suggest that in patients with DM and no DR, men may have a more inflammatory disease phenotype compared to women.

Prior studies have detected higher levels of numerous proteins associated with inflammation in the AH of patients with DM and varying levels of DR when compared to control groups.[15–18] Of the 12 proteins that were associated with male sex in patients with DM and no DR in the present study, monocyte chemotactic protein 2 has been identified as a potential biomarker

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Table 3. Select Results of Multivariate Linear Regression Analysis of Implicated Protein Biomarkers in Patients With DM With no DR

	Male Sex		Hemoglobin A1c	
Protein Biomarker	β	95% CI	β	95% CI
C-C motif chemokine 23	1.38	0.62 to 2.14	0.21	0.04 to 0.39
T-cell surface glycoprotein CD8 alpha chain	0.48	0.14 to 0.83	0.01	−0.07 to 0.09
Monocyte chemotactic protein 2	1.07	0.29 to 1.84	0.21	0.02 to 0.39
C-X-C motif chemokine 1	1.59	0.04 to 3.13	0.20	−0.16 to 0.57
Granzyme A	0.69	0.07 to 1.30	0.08	−0.07 to 0.22
Matrix metalloproteinase-12	1.10	0.37 to 1.84	0.13	−0.04 to 0.30
Matrix metalloproteinase-7	2.46	0.60 to 4.32	0.45	0.01 to 0.89
Monocyte chemotactic protein 4	0.91	0.30 to 1.52	0.22	0.07 to 0.36
C-X-C motif chemokine 10	1.68	0.03 to 3.32	0.41	0.02 to 0.80
C-C motif chemokine 17	0.85	0.05 to 1.65	0.14	−0.05 to 0.33
C-X-C motif chemokine 11	0.32	0.00 to 0.64	0.06	−0.02 to 0.14
TNF-related apoptosis-inducing ligand	0.69	0.03 to 1.35	0.08	−0.08 to 0.23

The dependent variable in the analysis was the NPX value for each protein biomarker. β coefficient 95% CIs that did not include zero were considered statistically significant. Results for age, systemic hypertension, hyperlipidemia, obesity, current smoker status, and insulin use are not presented; however, these covariates were included in the analysis as independent variables.

95% CI, 95% confidence interval; TNF, tumor necrosis factor.

for DR.[17] The remaining 11 proteins have not previously been implicated. This discrepancy may be due to limitations inherent to the proteomic platforms that were used in prior investigations. However, the possibility that these 11 proteins are not strongly associated with DM should also be considered.

It is now widely accepted that neuronal cell death is an early feature of diabetic retinopathy. This neurodegenerative process can result in functional changes that precede the development of obvious DR vascular lesions.[19] Multiple studies have demonstrated pathologic changes on microperimetry and multifocal electroretinogram studies and deficits on dark adaptation, contrast sensitivity, and color vision testing in patients with early DR.[20–23] Of note, this early neuroretinal dysfunction may manifest in a sex-specific fashion.[24] In a multifocal electroretinogram study of adult patients with type 2 DM and no DR, Ozawa et al.[25] found that local neuroretinal function was more abnormal in men compared with women. On the basis of these findings, the authors speculated that women may be more resistant to early neurodegenerative changes in DR because of superior tissue perfusion mediated by estrogen. Our results provide an additional explanation wherein a more inflammatory intraocular fluid milieu in men may play a role in these sex-based differences. Indeed, the theory that the accumulation of inflammatory proteins may contribute to retinal neuronal cell death in DM has already been proposed in prior reports.[10]

The underlying biological mechanisms for observed sex-based differences in DM, including our study's results, are not well understood. In general, men and women appear to be at higher risk for microvascular and macrovascular complications of DM, respectively.[4] Sex hormones are thought to play an important role in these discrepancies. For example, in the setting of ocular diseases associated with impaired blood flow, estrogen appears to be protective because of its reduction in large ocular vessel vascular resistance.[3] In contrast, higher levels of testosterone have been associated with DR incidence and severity.[26,27] In addition, gender-specific factors associated with health such as risky behaviors, work and home life expectations, health care access and utilization, and compliance with physician recommendations are also important.[1]

Personalized proteomics platforms have emerged as powerful tools to study vitreoretinal disease.[28] Multiple analytical methods exist ranging from shotgun mass spectrometry to multiplex immunoassays (MI). Mass spectrometry and similarly unbiased techniques are very sensitive and well suited for the identification of potential novel biomarkers. In contrast, targeted approaches such as MI can answer more focused questions in a relatively time- and cost-efficient manner

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with small sample volumes. In this study, we used a commercially available MI method based on an innovative PEA platform that allows for high-throughput analysis without loss of specificity. This technology has significant potential for translational investigations. For example, ocular biofluid proteomic analysis could be used to inform optimal treatment strategies based on the resultant biomarker profile. Indeed, this approach has already been used to study the relationship between AH inflammatory cytokine profiles and prognosis in patients with uveal melanoma.[29]

This study has several limitations. The sample size, particularly within each study group, was relatively small because of design constraints. Important clinical information such as duration of DM, menopause history, and estrogen use were not available in the electronic medical record for the majority of patients. Despite age and sex restriction in study group construction and the use of multivariate regression in our analysis, residual confounding because of unmeasured factors may have impacted our results. Last, potential biomarker concentrations were obtained through the use of a personalized proteomics approach and should be interpreted with caution. These dynamic proteomic profiles are the net result of all metabolic processes occurring at a given point in time and may not be related to DM or biological sex. As such, causal inferences should not be made in this setting. Given the exploratory intent of this study, we do not feel that these shortcomings invalidate our results. However, future work should be undertaken to confirm our findings in another independent cohort of samples.

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