

Metformin does not Reduce Inflammation in Diabetics with AAA or at High Risk of AAA Formation

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Introduction:

The protective effect of diabetes mellitus (DM) on abdominal aortic aneurysm (AAA) formation and growth has been repeatedly observed in population studies but continues to be poorly understood. However, recent investigations have suggested that metformin, a staple antihyperglycemic medication, may be independently protective against AAA formation and growth. Therefore, we describe the effect of metformin in AAA and at-risk patients on markers of inflammation, the driver of early AAA formation and growth.

Methods:

Peripheral blood was collected from patients previously diagnosed with AAA or presenting for their U.S. Preventive Task Force- recommended (USPTF) AAA screening. Plasma and circulating peripheral blood mononuclear cells (PBMCs) were isolated using ficoll density centrifugation. Circulating plasma inflammatory and regulatory cytokines were assessed with enzyme-linked immunosorbent assays (ELISA). CD4⁺ cell phenotyping was performed using flow cytometric analysis and expressed as a proportion of total CD4⁺ cells. To determine the circulating antibody to self-antigen response, a modified ELISA was performed against antibodies to collagen type V (COLV) and elastin fragments (ELNf).

Results:

Peripheral blood was isolated from 266 patients without DM (n=182), with DM not treated with metformin (n=34), and with DM actively taking metformin (n=50) from 2015-2017. We found no differences in the expression of Tr1, Th17, and Treg CD4⁺ fractions within diabetics ± metformin. When

comparing inflammatory cytokines, we detected no differences in IL-1 β , IL-6, IL-17, IL-23, IFN- γ , and TNF- α . Conversely, no differences were observed pertaining to the expression to regulatory cytokines IL-4, IL-10, IL-13, TSG-6, or TGF- β . Lastly, no differences in expression of COLV and ELNf antigen and/or antibodies were detected with metformin use in diabetics.

Conclusion:

Metformin in diabetics at-risk for AAA or diagnosed with AAA does not seem to alter the peripheral inflammatory environment.

Keywords: Abdominal Aortic Aneurysm, Inflammation, Diabetes, Metformin

Background

AAA is an major source of morbidity and mortality in the Western Hemisphere affecting as many as 4% of men starting their sixth decade of life.(1) This pathology is characterized by early local inflammation causing degradation of the arterial wall and progressive dilation until increasing wall tension eventually results in rupture.(2-4) Of those experiencing a catastrophic rupture event, approximately half die immediately and never present to the hospital; overall, short-term rupture mortality approaches 80%.(5, 6) In the United States alone, over 15,000 deaths per annum are directly related to AAA rupture making it the number 11 killer of Americans.(7)

The gold standard of AAA care continues to be rupture prevention via aneurysm resection and aortic reconstruction when cross-sectional diameter exceeds 5.5 cm.(8) As a result, over 40,000 highly-morbid aortic reconstructions are performed annually resulting in health care costs in excess of \$116,000 per case in the perioperative period.(9, 10) Therefore, the *magic bullet* for AAA continues to be the elusive pharmaceutical which can slow or stop diameter growth.

Previous population studies have suggested a protective effect of DM on AAA formation and growth.(6, 11-13) Only recently has metformin been implicated as a potential cause of the AAA-privileged nature of the diabetic patient.(14, 15) Our group has previously published studies suggesting the existence of an antigen-specific response unique to AAA subjects to breakdown products of elastin and collagen which may drive both immune aberrations leading to faulty regulation, via Tr1 and Treg dysfunction, and runaway inflammation, via Th17 overexpression.(16-19) Therefore, the purpose of this investigation was to establish the presence of cytokine changes, if any, in a population of AAA and high-risk patients associated with metformin use which may explain the potentially protective effect of metformin.

Methods

Blood Banking and Isolation of PBMCs and Plasma

After the appropriate approval was obtained from the Indiana University (IU) Institutional Review Board (IRB #1408881234), peripheral blood was collected from individuals giving informed consent at IU School of Medicine affiliated hospitals from 2015 to 2017. AAA-positive samples (>30 mm) were collected from individuals presenting for follow-up of previously diagnosed aneurysms while samples from at-risk subjects (risk-factor matched, RFM) were isolated from patients who screened negative for their USPTF recommended AAA ultrasounds at age 65.(20) Mononuclear cells were isolated by standard ficoll density separation using Accuspin tubes (Sigma) as described in detail elsewhere.(17) Isolated PBMCs and plasma were stored at -80 °C in small aliquots to minimize freeze/thaw cycles.

CD4⁺ Lymphocyte Phenotyping

Cell staining using antibodies against identifying surface markers to Tr1, Th17, and Treg subsets of CD4⁺ lymphocytes were performed per manufacturer's instructions (1:10, Miltenyi) unless otherwise noted. Tr1: CD4-FITC, CD49b-PE (1:20), LAG3-APC (1:20); Treg: CD4-FITC, CD25-PE, FOXP3-APC; Th17: CD4-FITC, CD194-PE, CD196-APC. Tr1 lymphocytes were stained for 15 minutes at room temperature while the remaining subsets were stained for 10 minutes at 4 °C. Flow cytometric analysis was performed on an Accuri C6 (BD) with CellQuest software (BD).

Plasma Cytokine and Antigen Quantification

Cytokine concentration in the plasma was determined using commercially available ELISA kits and performed per manufacturer's recommendations. Kits, when available, were obtained from R&D; if a cytokine kit was not available via R&D, an alternative was sourced from Sigma. To calculate absolute plasma concentrations, absorbance was read with a Cytation 5 (Biotek) at 450 nm and referenced to a standard curve.

Plasma Antibody Quantification

A modified ELISA was performed to determine relative concentrations of antibodies specific to COLV and ELNf. Human COLV (Sigma) and Elastin (Elastin Products Company) peptides were dissolved in phosphate buffered saline to a stock working solution of 25 ug/mL. This solution was used to coat a high protein binding 96-well polystyrene plate (Sigma) overnight at 4 °C. A blocking step was performed with a solution of 1% BSA for 2 hours at 37 °C or overnight at 4 °C. Plasma samples were sequentially diluted up to 1:1000 to determine optimal concentration and incubated for 2 hours at room temperature. A goat anti-human IgG Fc antibody conjugated to horseradish peroxidase (HRP, Sigma) was utilized as a secondary antibody per manufacturer's recommended dilution. Reactions were performed using a 1-step TMB turbo substrate (Sigma) for 30 minutes before a 1 M sulfuric acid stop solution was added. Absorbance was measured at 450 nm to determine relative intensity.(21)

Results

Baseline Comorbidities and Medications

From June 2015 to September of 2017, a total of 266 patients contributed blood to the biorepository utilized for this study. All patients, regardless of AAA status, were divided into three cohorts, 1) no DM (n=182), 2) DM without metformin (n=34), and 3) DM with metformin (n=50). The baseline comorbidities of these cohorts are detailed in **table 1**. We observed no difference with respect to age between cohorts ($p=0.98$). In terms of Framingham Risk Scores, nondiabetics had the lowest risk (33.1%) compared to a higher risk associated with DM regardless of metformin use (49.4% vs 49.8%). Our three cohorts were homogeneous in terms of AAA, chronic obstructive pulmonary disease (COPD), hyperlipidemia (HLD), coronary artery disease (CAD), and AAA familial history (FHx) incidence. However, heterogeneity was encountered with respect to the incidence of hypertension (HTN), peripheral arterial disease (PAD), chronic kidney disease (CKD), and obesity (BMI >30). Baseline medication profiles are described in detail in **table 2**. Although a significant difference in the usage of angiotensin-receptor blockers (ARB) was noted, this effect disappeared when the prevalence of angiotensin-converting enzyme inhibitors (ACEi) were combined. Except for aspirin use, medication homogeneity was encountered across all cohorts.

CD4⁺Lymphocyte Subsets

CD4⁺ lymphocytes of interest consisted of the regulatory Tr1 and Treg fractions and the inflammatory Th17 phenotype. We previously noted that patients with AAA demonstrated significantly decreased expression of the regulatory Tr1 (7.3% vs 1.4%, $p<0.01$) and Treg (2.6% vs 1.5%, $p=0.05$) lymphocytes compared to their RFM counterparts.(4, 17) Additionally, a strong trend suggesting an

increase in the expression of the inflammatory Th17 cell was observed in the patients diagnosed with AAA. Therefore, we investigated whether the expression of these cell types normalized with the presence of metformin. In the diabetics, we did not observe a change in the Tr1 (3.0% vs 5.0%, $p=0.24$), Th17 (3.0% vs 2.9%, $p=0.92$), or Treg (1.6% vs 1.2%, $p=0.47$) fractions with the presence of metformin which would suggest a beneficial effect on decreasing inflammation (**Figure 1**).

Inflammatory Cytokines

The effect of metformin on the inflammatory cytokines OPN, IL-1 β , IL-6, IL-17, IFN- γ , TNF- α , and IL-23 were examined. The presence of DM did not seem to significantly alter the inflammatory environment compared to nondiabetics. Although weak trends towards decreased concentrations of OPN (9.1 vs 6.3 ng/mL, $p=0.34$), IL-1 β (4.4 vs 3.9 ng/mL, $p=0.45$), and IFN- γ (18.4 vs 9.2 ng/mL, $p=0.24$) were seen, no statistically significant reduction in inflammatory cytokines were confirmed with the introduction of metformin to diabetics (**Figure 2**).

Regulatory Cytokines

The regulatory cytokines TGF- β , IL-4, IL-10, IL-13, and TSG-6 were used as markers of inflammation suppression (**Figure 3**). While IL-4 concentration increased in response to metformin (2.7 vs 5.1 ng/mL), this did not reach statistical significance ($p=0.23$). In the remaining regulatory cytokines examined, no trends were noted to suggest an antiinflammatory effect of metformin in diabetics taking metformin.

COLV and ELNf Antigen and Antibody

In our previous experiments, we noted significantly higher concentrations of both ELNf/COLV antigen and antibodies in circulation of RFM and AAA patients compared to healthy controls. However, we did not observe an inflammation suppressing effect of metformin on circulating COLV (0.73 vs 0.62 ng/mL, $p=0.56$) or ELNf (15.1 vs 14.8 ng/mLs, $p=0.94$) antigens. Between all cohorts, plasma antigen levels were lowest in the nondiabetic patients. Similarly, antibodies specific to COLV (0.42 vs 0.38 Relative Units, $p=0.76$) and ELNf (0.22 vs 0.20 RUs, $p=0.78$) did not differ with the introduction of metformin. With respect to both peptides, a slight nonsignificant increase was observed in circulating antigens and antibodies with the presence of DM compared to patients without DM.

Discussion

We established the IU Center for Aortic Disease biorepository in 2015 as part of a concerted effort to stimulate research into the immune component of AAA formation and growth. Since the beginning of this initiative, we have collected blood from over 330 individuals. With the assistance of this growing collection, multiple anomalies in the human immune response that characterizes the AAA condition have been described.(17, 19)

The negative association between DM and AAA has been observed and noted in numerous population-based studies over the previous two decades.(15) A recent meta-analysis pooled 13 population, prospective cohort, and case-control studies and reported an odds ratio of 0.59 (95% CI, 0.52 – 0.67; $p<0.01$) in support of the protective effect of DM on AAA formation.(22) Another similar review identified 17 large population studies and observed an odds ratio of 0.80 (0.70 – 0.90; $p<0.01$) between DM and AAA once again implicating DM as a protective factor.(12) It is not clear how DM attenuates AAA formation; however, animal models of hyperglycemia have demonstrated evidence of decreased infiltration of macrophages, elastolysis, and neovascularization of the aortic wall which are all hallmark of early AAA formation.(23) Additionally, the diabetic environment may also favorably alter vascular smooth muscle cell physiology, increase advanced glycation end-products, down-regulate matrix metalloproteinases, and decrease fibrinolysis causing an overall increase in aortic structural integrity.(24)

With the increasing drive to establish a pharmaceutical treatment for AAA, the role of medications associated with DM have been scrutinized in detail.(25) These initial studies supported a protective effect of metformin, thiazolidinediones, and sulfonylureas not observed in α -glucosidase and DPP-4 inhibitors.(25) While the effect of thiazolidinediones and sulfonylureas have been called into question by some studies claiming little protective effect, metformin seemed to be particularly effective at

both prevention and progression of AAA across multiple clinical investigations.(14, 26) It is thought that metformin functions to decrease circulating glucose by inhibiting hepatic gluconeogenesis and blunting the effect of glucagon via inhibition of mitochondrial complex I and inactivation of the protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) signaling pathway.(27) This effect also results in an increase in overall sensitivity to circulating insulin. While metformin's AAA-protective mechanism continues to be nebulous, suggestions of a pleiotropic effect consisting of the inhibition of extracellular matrix (ECM) remodeling, downregulation of inflammation, and reduction of oxidative stress have been made.(14, 28-31)

We did not observe a decrease in inflammation with metformin use in a combined cohort of at-risk and AAA patients. However, when the population is divided into AAA and non-AAA patients by metformin status, no differences are once again seen with respect to CD4⁺ lymphocyte, cytokine, antigen, and antibody expression.

We previously reported significant aberrations between the RFM and AAA populations with respect to CD4⁺ fractions of lymphocytes in circulation.(17, 19) In particular, there is a depletion of the Tr1 (CD4⁺LAG3⁺CD49b⁺) regulatory lymphocyte in the AAA condition.(17-19) This inflammation suppressing cell is unique in the immune response as it is antigen specific, elaborating the regulatory cytokine IL-10 and eliminating myeloid derived antigen-presenting cells via the granzyme B pathway in response to antigen recognition and activation to suppress local inflammation.(32) Not surprisingly, we also noted a reduction of circulating IL-10 in AAA patients corresponding to the aforementioned loss of Tr1 activity.(17) In this study, we did not observe an antiinflammatory effect of metformin on IL-10 or Tr1 expression towards levels seen in the RFM population. Additionally, no therapeutic effect was seen in the inflammatory Th17 or regulatory Treg population towards "healthy" levels.

Previous reports suggest that circulating elastin and collagen type V peptides may be significant antigens in the pathogenesis of COPD, an inflammatory and destructive disease of the pulmonary system and strong risk factor for AAA formation.(21, 33, 34) In our AAA population, we noted increased expression of both soluble elastin antigen and antibodies circulating in the peripheral plasma of AAA patients compared to RFM controls.(4) We believe these two antigens may play a major role in driving the self-sensitization of inflammatory immune cells to the native infrarenal aortic wall as they predictably increase from healthy, to RFM, and finally AAA-diagnosed subjects.(4) Once again, our investigation did not demonstrate a decrease with metformin in the circulation of these inflammatory peptides in the diabetic population.

Although previous metformin studies suggested a reduction in the rate of AAA formation and growth, no benefit was observed with on the risk of AAA rupture. A recent study from Denmark reviewed a national ruptured AAA registry over 15 years starting in the late 1990s (n=362). In their series, 22.4% of ruptures were long-term metformin users (compared to 28.8% of controls). After adjustment for covariates, the OR reported was 0.84 (CI, 0.61 – 1.17) suggesting no effect of metformin in the diabetic population at rupture risk reduction.(35) The authors conclude their findings, in conjunction with previous studies establishing the effectiveness of all antidiabetic medications equally(36), suggest that DM, rather than metformin, is the overwhelming variable generating protection to AAA. The data presented in this manuscript can be interpreted to provide limited evidence supporting that assertion. However, it is worth noting that with exception to Treg (higher in nondiabetics) and Th17 expression (lower in diabetics), no differences were noted in the markers reported in this manuscript when the RFM cohort was compared to diabetics regardless of metformin status as well.

There are several limitations to the results reported in this study. Because of the design of this investigation, a retrospective review of a prospectively maintained database, small numbers of patients

were included in several of the groups for statistical analysis. This limits the power, amplifies error, and makes conclusions weak. Additionally, the vast majority of samples in our biorepository was collected from patients at our VA medical facility; therefore, nearly 100% of the subjects studied were male further limiting the generalizability of the data presented.

Conclusion

Although metformin has been associated with protection against AAA in previous studies, we did not observe any alterations in cytokine signaling, CD4⁺ phenotype expression, or circulating antigens/antibodies which would explain this effect.

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