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Morphometric and histological parameters in veins of diabetic patients undergoing brachiocephalic fistula placement

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Abstract

Diabetic patients with end-stage renal failure have higher rates of arteriovenous failures when compared with nondiabetics. The aim was to compare differences in indicators of vascular remodeling and endothelial dysfunction in veins of patients with or without diabetes at the time of surgical placement. In this prospective observational trial, vein samples were collected from patients when a brachiocephalic fistula was created. Morphometric measurements and extent of fibrosis were determined using Image J software. Histological analysis, for the presence of myofibroblasts and level of endothelial nitric oxide synthase, was performed by immunohistochemical staining and scored in semi-quantitative manner. Asymmetric dimethylarginine was determined at the time of access placement. Comparison of diabetics and nondiabetics was performed using Wilcoxon rank sum and Fisher's exact tests. Eighteen patients were included; 10 were diabetics. There was a significant difference in the measurement of vein area between groups, with diabetic vein samples having larger luminal area of average 832,001.18 μm^2 (317,582.17–3,695,670.36, $P = 0.04$). The maximal intimal to medial thickness ratio was higher in diabetic vein samples (0.71 vs. 0.24, $P = 0.03$) along with statistically significant higher maximal intimal thickness (312.12 vs. 115.14 μm , $P = 0.03$). There is a significant difference in vascular wall remodeling between diabetics and nondiabetics at the level of the cephalic vein at the time of brachiocephalic placement. The unexpected finding of significantly larger luminal area in diabetic veins could be a major factor positively affecting brachiocephalic outcomes in otherwise impaired remodeling in this patient population.

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DISCLOSURES

None.

Keywords

Fistula; diabetes; vascular disease; intimal hyperplasia

INTRODUCTION

Diabetes is a primary cause of accelerated vascular disease causing significant impact on morbidity and mortality in this patient population.^{1,2} The pathogenic lesion of diabetic arterial disease begins with endothelial cell injury, involves smooth muscle cell activation, and is characterized by calcific plaque, ferritin deposition, and eventual intimal hyperplasia.^{1,3,4} Atherosclerosis in diabetes has been well characterized in the arteries although the pathology of diabetic venous disease is not well described.⁵

The primary cause of end-stage renal disease (ESRD) is diabetes, with arteriovenous fistula (AVF) required for chronic hemodialysis. When an AVF is created, it is important that the vein undergo an arterialization process that may take months before the fistula is ready to use for dialysis. If a vein does not successfully dilate, the access will fail which happens commonly in diabetics with lower arm fistulas.^{6,7} Defective remodeling has been shown to occur in diabetics and contributes to these poor outcomes.⁸ Because of an early and significant failure rate in lower arm AVF, upper arm fistula, specifically the brachiocephalic (BCF) configuration, is used more frequently in diabetics.⁷

Intimal hyperplasia (IH) is the primary mechanism of AVF failure. Intimal hyperplasia is a tissue response to injury because of inflammation, oxidative stress, and endothelial dysfunction.^{8,9} All are highly elevated in advanced renal failure patients as well as presence of diabetes. It is unclear which cellular and metabolic changes lead to changes in the vein architecture and resultant IH. Given the aggressive vascular predisposition of diabetes, our hypothesis is that endothelial dysfunction, as a result of diabetes, is present in veins at the time of AVF creation, contributing to the adverse outcome in this patient populations.

Outward vasculature remodeling is important in AVF maturation. One of the important mediators of vascular remodeling is nitric oxide (NO). In normal vessels, increased blood flow, such as what occurs with a fistula creation, stimulates endothelial cells to produce endothelial nitric oxide synthase (eNOS) with subsequent production of NO and an increase in luminal diameter. NO is also a negative regulator of vascular smooth muscle cell proliferation. If adequate eNOS is decreased, vascular dilation may not occur and remodeling may be abnormal.¹⁰ Another protein important in the remodeling process is asymmetric dimethylarginine (ADMA). This protein is elevated in renal failure and has been shown to directly inhibit NOS, contributing to intense vasoconstriction and endothelial dysfunction.¹¹ The influence of the eNOS/ADMA pathway on vasculature remodeling in veins is not well understood.

The aim of the current project was to evaluate the morphometric differences and histological parameters in vein sections of patients without and with diabetes taken at the time of BCF placement. A secondary aim was to determine the influence of the NO system on vascular remodeling by measuring eNOS by immunohistochemistry and serum values of ADMA. A

correlation was made with fistula outcomes and determined if diabetic control, blood pressure control, parathyroid hormone levels, phosphorus levels, and dialysis days prior to the access placement played a role. Insight into the pathology of veins in real failure will allow for an improved understanding of the vascular disease in diabetes.

MATERIALS AND METHODS

This project was part of a large prospective, observational cohort study evaluating fistula outcomes and was conducted at a large, single-center university-based medical center from October 7, 2011 to January 22, 2014. Briefly, patients were included in the study if it was determined that a primary BCF fistula was to be placed for dialysis vascular access, with no previous access in the ipsilateral arm. The subjects gave written consent as approved by the Institutional Review Board at the University of Chicago Medical Center. Venous tissue specimens were collected at the time of surgical creation of vascular access. During the surgery, a circumferential segment of vein was removed near the planned anastomosis site in each patient and immediately fixed in formalin. Each venous tissue sample, fixed in formalin, was embedded and cut into eight tissue blocks of 3–4 mm thickness. Each piece was paraffin-embedded and then sliced into 4 μm sections for histological and immunohistochemical studies. The slides were reviewed from April 1, 2014 to July 31, 2014 by one pathologist (AC) and one nephrologist (IL), who were both unaware of the clinical information and AVF outcomes, for morphometric and histological analysis.

Patient characteristics

Patient's demographics and clinical parameters of interest include: age at time of surgery, history of diabetes, hemoglobin A1C greater than or equal to 8, dialysis days prior to access placement, average blood pressure greater than 140/90, average parathyroid hormone greater than 600 pg/mL, and average phosphorus greater than 5 pg/mL over the past year.

Morphometric analysis

Morphometric analysis was performed using Image J software (National Institute of Health, Bethesda, Maryland, USA) on images taken from cut paraffinized vein samples using Masson's trichrome stain. Direct measurements and calculated parameters obtained included: internal and external luminal perimeter, calculated diameter ($\text{perimeter}/\pi$), luminal, intimal and medial area; ratio of intimal to medial area; percentage of luminal stenosis; maximal intimal and medial thickness; mean intimal to medial thickness ratio, and maximal intimal to medial thickness ratio. Perimeter measurements were made of the internal and external circumference of the vein and results (in pixels) were multiplied by 2.00012 (conversion factor for pixels to micrometers) and expressed as micrometers (μm). Area measurements as depicted in Figure 1 were made of the luminal area (A), area enclosed by the intima (B), and area enclosed by the media (C). Luminal area, intimal area, and medial area were multiplied by 4.46337 (conversion factor for pixels to micrometers) and expressed in units of micrometers squared (μm^2). Percent of luminal stenosis was calculated using the formula $(1 - A/C) \times 100$. The ratio of intimal area to medial area was calculated using the formula $(B - A)/(C - B)$. To obtain a representative measurement of intimal and medial thickness, a point was placed in the center of the lumen and was used to

obtain a four quadrant grid (0, 90, 180, and 270). At intersecting points with intima and medial, measuring was obtained at the 90 degree angle. All intimal thickness, medial thickness, maximum medial thickness, and maximal intimal thickness were multiplied by 2.11267 (conversion factor for pixels to micrometers) and the results were expressed in units of micrometers (μm). Results were derived and intima-media thickness mean and ratio were calculated. This approach was previously described by another group.¹²

Histological analysis

Standard immunohistochemistry was performed using smooth muscle actin (SMA; M0851, clone: 1A4, dilution 1:100, DAKO, Carpinteria, CA, USA), caldesmon (M3557, clone: h-CD, dilution 1:50, DAKO), and calponin (M3556, clone: CALP, dilution 1:50, DAKO) to identify myofibroblasts (SMA/calponin positive and caldesmon negative) and smooth muscle cells (calponin negative and caldesmon/SMA positive).^{13–16} Samples were scored separately by one pathologist (AC) using a semi-quantitative score from 1 to 4+. Endothelial nitric oxide synthase immunohistochemical staining was used to assess NO as a marker of oxidative stress in the intima. For eNOS immunohistochemical analysis, samples were incubated with polyclonal rabbit antibody against nonphosphorylated eNOS (BD Transduction Laboratories, San Jose, CA, USA) at a dilution of 1:20. Placental tissue was used as a control. The presence of eNOS in circumferential endothelial wall was evaluated using a scoring system by one pathologist (AC) and one nephrologist (IL) on a semi-quantitative scale (0 = less than 30% of endothelial cells positive; 1 = 30–60% of endothelial cells positive; 2 = greater than 60% of endothelial cells positive). The immunohistochemistry examination of eNOS was also scored as negative and positive. Masson trichrome stain was used to determine the degree of intimal or medial fibrosis with collagen staining blue and smooth muscle cells staining red. Image J software using split image feature in three color channels, measuring color saturation, was used to determine the percentage of collagen deposition.

ADMA

In each patient, a 10-mL blood sample was drawn preoperatively in a recumbent position the day that the fistula was placed. The blood samples were centrifuged at 3000 rpm for 15 minutes at 4°C immediately after collection. The plasma samples were then stored at –80°C until use. Sample derivatization and chromatography were performed as previously described by Sotgia et al.,¹⁷ with minor adaptations, to measure the concentration of ADMA in plasma samples. Briefly, 2.5 μL of 5mM homoarginine (internal standard) was added to thawed plasma samples. This mixture was treated with 200 μL of acetonitrile to precipitate the larger proteins and centrifuged at 7000 rpm for 7 minutes. Twenty microliters of 5% KOH and 20 μL of 2% w/v ninhydrin ethanolic solution were added to the supernatant from the above step (derivatization step) and then incubated at room temperature in the dark for 5 minutes. Twenty microliters of 5M H_2SO_4 was added to neutralize the solution and was incubated at 100°C on a heating block (color development step). The sample/mixture was vortexed between each step to ensure proper mixing. All reagents used for derivatization were prepared in ultrapure deionized water.

Chromatography was performed using an Agilent (Santa Clara, CA, USA) LC system model 1200. Separation was achieved by using a 4.6 mm × 15 cm Agilent Extend C18 5 µm column with a 4.6 mm × 20 mm guard column cartridge. The mobile phase, an aqueous solution of 13 mM ammonium bicarbonate (NH₄HCO₃) with 10% (v/v) tetrahydrofuran, was delivered isocratically across the column at 1.3 mL/minute at room temperature (22–23°C). Twenty microliters of the derivatized sample was injected and the fluorescence signal was recorded with the excitation and emission wavelengths set at 390 and 497 nm, respectively, and gain set at ×10 scale expansion. Data were recorded for 17 minutes for each sample. The peak area of the internal standard, the retention time for which was 5.5 minutes, was used to extrapolate the concentration of ADMA, the retention time for which was 11.5 minutes.

Outcomes

For each patient, the date of access placement was taken as the baseline and the clinical course was followed for up to 24 months. Outcomes were analyzed as combined failure endpoint of cephalic arch stenosis, thrombosis, or venous outflow stenosis. These outcomes were also analyzed individually.

Statistical analysis

Continuous variables were summarized with medians and full ranges (minimum and maximum values). Counts and percentages were used to summarize binary variables. Wilcoxon's rank sum test and Fisher's exact test were used to compare continuous and binary characteristics (respectively) in subjects who did or did not experience failure within 6 months. Given the small sample size, univariate analyses were used to show the association between measured parameters and diabetes or outcomes. The Benjamini-Hochberg method of P value adjustment was used to control the false discovery rate at 5%. All statistical analyses were performed in R version 3.0.2 (<http://www.r-project.org/>).

RESULTS

Patient characteristics

Table 1 shows the main characteristics of the 18 patients that had vein specimens with a circumference that enabled accurate measurement of the intraluminal perimeter, area, and other histologic parameters of interest. There was no significant difference between diabetics or nondiabetics with respect to blood pressure control, PTH, phosphorus control, age, or days of dialysis.

Morphometric analysis

J-image analysis was performed at the University of Chicago by one of the investigators (IL), who was trained in this technique and blinded to the identity of the tissue. Table 2 shows the histomorphometric measurements in diabetic and nondiabetic subjects. Diabetics had a significantly greater internal and external luminal perimeter, luminal, intimal and medial area, maximal intimal thickness, intimal to medial ratio, and intimal to medial thickness ratio when compared with nondiabetics (Figures 2 and 3). The diameter of the vein at the antecubital fossa measured by venous duplex Doppler was available in 11 subjects

preoperatively. The actual measured diameter did not correlate with the calculated internal diameter ($r = -0.55$, $P = 0.6$) or calculated external diameter ($r = -0.1$, $P = 0.92$).

Histological analysis

Semi-quantitative analysis was scored at the University of Chicago by one pathologist (AC) blinded to the identity of the tissue. Table 3 shows the staining scores for diabetics vs. nondiabetics. There were no significant differences between the two groups. Figure 4 shows representative vein sample for stain with caldesmon, calponin and SMA.

eNOS and ADMA

While diabetics had a higher semi-qualitative score for eNOS (44.4% with greater than 60% of circumferential endothelial cells stained) compared with nondiabetics (22.2%), there was no statistical significance between the three groups. The average ADMA concentration was $2.061 \mu\text{mol/L} \pm 0.759$ for all 18 subjects, with the highest being $3.9952 \mu\text{mol/L}$ and the lowest being $0.9006 \mu\text{mol/L}$. There was no significant difference found between diabetics ($2.05 \mu\text{mol/L}$) and nondiabetics ($1.8 \mu\text{mol/L}$) ($P = 0.80$). Figure 4 shows representative vein sample for stain with eNOS.

Outcomes

Most of the patients (13 of 18) experienced combined failure, defined as venous outflow stenosis, cephalic arch stenosis, or thrombosis, with follow-up of at least 6 months. There were five episodes of thrombosis, all in diabetic patients which was significant when compared with nondiabetic patients ($P = 0.036$). Table 4 shows histomorphometric measurements for patients with and without a thrombotic event. Intimal area was significantly greater in thrombotic vs. nonthrombotic outcomes ($P = 0.03$). Staining scores for myofibroblasts, eNOS, or trichrome did not predict evidence of failure (data not shown).

CONCLUSION

The internal area of the vein used to create BCF was found to be greater in diabetics when compared with nondiabetics. In particular, the internal lumen, intimal, and medial area were all found to be more dilated in diabetics. As the luminal area is influenced substantially by the way the tissue is embedded, the perimeter of the veins samples was measured. The internal and external perimeters of the veins were found to be greater in diabetics that support the larger luminal area in diabetics. Our findings are the first report of this morphometric parameter difference in diabetics. This altered remodeling may be beneficial as previous reports have shown that venous stenosis is attenuated in diabetics with BCF.¹⁸

The primary histological abnormality responsible for stenosis is the development of IH. Intimal hyperplasia was found to be greater in diabetics than nondiabetics, a finding similar to previous reports.¹⁹ A thrombotic event was also found to be more common in diabetics and may be related to increased intimal area. It has been found that venous IH may preexist in the veins used for fistula construction in patients with advanced chronic kidney disease (CKD) predisposing patients to access failure.^{19–21}

Current practice for preoperative fistula evaluation often includes Duplex vein mapping to establish adequate vessel diameter. Vein diameter has been shown to predict functional AVF maturation;²² however, one study found no difference between diabetics and nondiabetics.²³ In the current study, the diameter of the vein was calculated from the internal and external perimeter ($\text{perimeter}/\pi$) and was compared with the diameter of the vein at the antecubital fossa estimated by mapping venous Doppler in a subset of patients. There was no statistical correlation found. This is not surprising as the Doppler measurements were performed preoperatively up to 1 month prior to AVF placement and not on the day of the surgery. We acknowledge that we have a very small sample size which limits the validity. An adequate tool available to predict suitable veins for a mature AVF is needed. If we are able to identify the optimal diameters of vein and arteries with suitable histology for AVF creation, we may have better success at fistula outcomes. Future trials measuring intra-operative vein diameter and blood flow and comparing this with the extraluminal Doppler is required to better define the actual vessel parameters which are optimal for AVF maturation.²⁴

Endothelial dysfunction and activation is an indicator of vascular disease.²⁵ Advanced CKD and ESRD patients have impaired endothelial function attributed to increased oxidative stress and inflammation.²⁶ Increased oxidative stress renders eNOS dysfunctional such that endothelial cells do not produce NO.^{27,28} Endothelial cells may be activated with increased blood flow such as that occurs when a fistula is created. One of the culprits for endothelial dysfunction is decreased NO production. ADMA, a known regulator of NO, has been linked to fistula failure.²⁹ Given that ADMA is shown to cause intense vasoconstriction while NO is a vasodilator, one hypothesis was that eNOS would be increased and ADMA would be decreased in the veins with a larger intraluminal area. As expected, ADMA was found to be elevated uniformly with an average value of 2.0606 $\mu\text{mol/L}$. This could be due to renal failure as normal controls have a reference value of 0.43–0.96 $\mu\text{mol/L}$.³⁰ Our data show a marginal increase in plasma ADMA levels in patients with diabetes as compared with nondiabetics which was not significant. A second hypothesis was that, given the known vascular disease in diabetics, they would show more oxidative stress and less eNOS expression. We observed a 50% increased expression of eNOS in the diabetic patients, which was not statistically significant because of the small sample size analyzed in this study.

Vascular wall remodeling differs between diabetics and nondiabetics with increased IH in the former.³¹ As IH increases, this can dramatically decrease the lumen size and can lead to a negative vascular remodeling and vasoconstriction. Most past research focuses on IH although outward remodeling is an equally important process that could preserve the vein lumen and predict the outcome of the fistula over time.³¹ This critical balance between IH and outward remodeling in a vein with a created fistula is important.

Why is this dramatic venous dilatation evident in diabetics? We postulated that maybe the veins of diabetics were more fibrotic or calcified with resultant fixed dilation. Trichrome immunohistochemistry did not support increased collagen deposition in diabetics. Microcalcification has been shown to be increased in arteries used to create AVF.^{3,32} We attempted immunohistochemical stain with von Kossa in all samples to detect microcalcification and none of the samples had large deposits in the vessel profiles. The

calcification was also not prominent in the hematoxylin and eosin, so we conclude in this preliminary study that either the stain is not representative of calcification or the vein is not calcified to a significant degree to account for the larger area observed in diabetics.

Limitations to this study include a small sample size with a longer follow-up needed for all subjects. We were limited to include only those vein samples with complete circumference, and therefore the results of this study will need validation with a larger number of samples. The semi-quantitative scoring method may also have subjective error although one pathologist and one nephrologist each scored the samples independently. The pathway for NO and ADMA production is complex with many intermediates which need to be quantified before a conclusion is reached as to the interplay this pathway could have on the endothelium. There are many other factors, such as transcription factor E26 transformation-specific sequence-1, known to mediate IH and defective remodeling which were not measured in the current study and are of interest for future investigation.³³

In summary, the venous luminal area is larger in diabetics when compared with nondiabetics which is an unexpected finding and might be contributing to better BCF outcomes in this patient population. The vascular response causing this finding is not elucidated in this study. We found no significant difference in endothelial dysfunction and extracellular matrix deposition as well as cellular phenotype pattern between diabetics and nondiabetics. The current approach to placement of a vascular access includes preoperative Doppler and or venogram to determine suitable arterial/vein diameters. It may be that the intraluminal area may be a better predictor as to the adequacy of vein maturation for fistula access. Further research to characterize the intraluminal surface area and endothelium using such tools such as intravascular ultrasound will give important insight into the mechanism of fistula failure with identified possible treatment options.

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Figure 1. Morphometric area measurements of a vein sample, Masson's trichrome stain of a representative sample from a circumferential vein. Boundary area is represented by solid black line. A represents the intraluminal area, B represents the intimal area, and C represents the medial area.

Figure 2. Histology of vein specimen of a patient without diabetes. Hematoxylin and eosin stain of a representative sample from a vein collected from a nondiabetic patient.

Figure 3. Histology of vein specimen of a patient with diabetes. Hematoxylin and eosin stain of a representative sample from a vein collected from a diabetic patient. Note the large intraluminal area.

Figure 4. Immunohistochemical stains of vein. Smooth muscle bundles are highlighted in brown for vein sample 40 $\times$ by caldesmon (A), calponin (B), and smooth muscle actin fibers (C). Vein sample 100 $\times$ with endothelial nitric oxide synthase stain (D) shows dark brown endothelial cell border.

Table 1

Patient characteristics of nondiabetic and diabetic patients

Variable	Nondiabetic n = 8	Diabetic n = 10	P value
Blood Pressure control	2 (25%)	4 (40%)	0.85
Phosphorus (mg/dL)	3 (37.5%)	4 (40%)	1.00
Age (years)	54.5 (24–84)	62 (45–91)	0.85
Dialysis days (years)	1.5 (0–21)	2.25 (0–60)	0.85

Table 2

Histomorphometric measurements of nondiabetic and diabetic patients

Measurement	Nondiabetic n = 8	Diabetic n = 10	P value
Internal perimeter (μm)	4252.95 (1567.76–6539.8)	7099.94 (3224.6–10502.57)	0.02
External perimeter (μm)	8745.3 (6944.54–11487.88)	11270.76 (9891.34–16377.72)	0.02
Lumen area (μm ²)	514003.92 (52453.52–786570.77)	832001.18 (317582.17–3695670.36)	0.04
Intimal area (μm ²)	919920.64 (164783.16–1383791.99)	1896519.39 (1288088.41–7696590.59)	<0.001
Medial area (μm ²)	2772741.4 (1425832.47–7552588.89)	5041043.89 (2619636.66–15888298.36)	0.04
Maximal intimal thickness (μm)	115.14 (52.82–297.89)	321.13 (139.44–807.04)	0.03
Maximal medial thick (μm)	473.24 (348.59–847.18)	428.75 (154.22–935.91)	0.89
% lumen stenosis	86.33 (74.96–96.32)	78.56 (44.88–91.11)	0.39
Intima/medial area	0.12 (0.07–0.54)	0.44 (0.09–1.8)	0.04
Intima/medial thickness mean	0.13 (0.1–0.79)	0.53 (0.07–1.99)	0.10
Intima/medial thickness ratio	0.24 (0.14–0.76)	0.71 (0.25–2.44)	0.03

Table 3

Staining scores of nondiabetic and diabetic patients

Stain	Nondiabetic n = 8	Diabetic n = 10	P value
Myofibroblasts	5 (62.5%)	5 (50%)	0.79
eNOS			0.79
0	1 (5.6%)	1 (5.6%)	
1	3 (16.7%)	1 (5.6%)	
2	4 (22.2%)	8 (44.4%)	
eNos (+)	6 (75%)	7 (70%)	1.00
Intimal trichrome %	50.71 (16.46–64.39)	57.24 (12–77.99)	0.79
Media trichrome %	28.71 (16.57–42.71)	16.66 (6.64–37.61)	0.60

eNOS = endothelial nitric oxide synthase.

Table 4

Histomorphometric measurements of patients without and with thrombosis

Measurement	Nonthrombosis n = 13	Thrombosis n = 5	P value
Lumen area (μm^2)	6974555.12 (52453.52–2993006.48)	504445.61 (317582.17–3695670.36)	0.77
Intimal area (μm^2)	1275372.27 (164783.16–4845854.03)	1995153.17 (1516599.57–7696590.59)	0.03
Medial area (μm^2)	3235715.62 (1425832.47–7552588.89)	5440700.74 (2619636.66–15888298.36)	0.30