Investigating Macrovascular Wall Thickness of the Forearm in Systemic Sclerosis

Master Research Project 1

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Abstract

The auto-immune inflammatory disease systemic sclerosis is still relatively unknown. Research concerning the macro vascular of the forearm is scarce. Investigating the vasculature of the forearm may give us better insight in the progression of the disease. The aim of this research was to look for possible abnormalities of the vessel wall in the brachial, ulnar, and radial arteries in systemic sclerosis patients. Forty-two patients and twelve healthy controls were studied with a median age of 62 and 58 respectively. Intima-media thickness (IMT) and lumen diameter were measured using ultra-high frequency ultrasound (48 MHz). A severity score index was formulated and applied to include unmeasurable ultra-sound pictures in the analysis. Results showed higher severity scores for stenosis or occlusion in the ulnar arteries compared to the other vessel (21,4% and 16,7% versus 2,4% for the right brachial and 0% for the radial arteries and left brachial). No significant differences were observed for the IMT. The SSc group has significant decreased lumen diameter for the radial and ulnar arteries ((p=0.042), (p=0.001), (p=0.001) and (p<0.001) respectively). This research has shown that defining the borders of the adventitia-media-intima is essential to get relevant and precise IMT scores. Moreover, this research project has given light to many opportunities for follow-up research. Increasing the list of study variables can clarify inconsistencies in the data. Investigating the relation with different clinical parameters like, capillaroscopy and calcinosis can be possibilities for additional research as well. Despite its exploratory nature, this research project's outcomes support the notion that the macrovasculature (radial artery and ulnar artery) are involved in the progression of SSc and can provide support for future research and interventional treatment options regarding SSc.

Keywords

Systemic sclerosis, intima-media thickness, macrovascular disease, stenosis, occlusion

Introduction

Systemic sclerosis (SSc), also called scleroderma, is an auto-immune inflammatory and fibrotic connective tissue disease (1). The disease cause and pathways are relatively unknown and treatment options are limited (1). It is categorized by fibrosis of the skin and internal organs as well as microvascular abnormalities (1). Two patterns of SSc exist: limited cutaneous and diffuse cutaneous. The former is characterized by sclerodactyly, telangiectasia, esophageal dysfunction, calcinosis, and Raynaud's phenomenon (RP) (1,2). Next to these features, the diffuse subtype is also associated with an increased risk of cardiovascular disease, acute renal injury, and pulmonary complications (1,2,5,6). Although systemic sclerosis is uncommon, it has high relative high mortality rate with an estimated mortality ratio (SMR) between 2.5 and 4 (3,4). It is important to note that SSc is a risk factor for early peripheral arterial disease of both the upper and lower extremities (7). A large cohort study indicated a four times higher overall mortality rate in SSc patients, as compared to the general population, and one-third of these deaths are associated with cardiovascular pathologies (8).

The vasculopathy of the disease is considered to be mainly affecting microvasculature of the body. Currently the main diagnostic tool to measure microvascular damage in SSc is nailfold capillaroscopy (NFC). During this examination high-resolution images are made and assessed for (abnormal) capillary characteristics and density. These assessments can be classified in SSc patterns ('early', 'active', 'late') and can be summarized in the "microangiopathy evolution score" (MES) (9,10). However, recently research has shifted focus to abnormalities in the macrovasculature progression in SSc. For instance, Hughes et al. (2021) has shown an increased vessel wall volume in the distal arteries in SSc patients compared to healthy controls (11). Also, increased arterial stiffness and plaque presence was reported which are both indicators of macrovascular involvement (12,13). Unfortunately, conflicting results still exist concerning the intima-media thickness (IMT); which is a way of quantifying vessel wall thickness, another possible indicator of macrovascular disease, of larger vessels and its correlation with the progression of SSc. A systematic review by Nussinovitch and Shoenfeld (2011) discussed IMT differences in SSc patients and controls. All these studies, however, were limited to the carotid artery, as it is correlated with traditional risk factors of atherosclerosis. The studies reporting differences in IMT showed significant results, whereas the studies reporting the opposite did not. Furthermore, other studies indicated a positive correlation with the progression of SSC and endothelial dysfunction and increased carotid IMT (14,15).

Interestingly, minimal research has been conducted regarding the involvement of the vasculature of the forearm in SSc (i.e., brachial, radial, and ulnar artery) (6,16). A prospective study in 2017 showed that up to 25% of SSc patients may develop an ulnary artery occlusion, a macrovascular feature which is far more frequent in SSc than in the general population, which can even lead to severe complications in the digits (17,18). When looking at several computerized tomography angiographic features like occlusion and/or stenosis, the brachial artery has been shown to be afffected in SSc irrespective of the disease pattern. The radial and ulnar artery have also been shown to be involved in the progression of macrovascular disease (19,20).

As previously published research only focused on the vessel wall of the carotid artery. The brachial, radial, and ulnar arteries have not been properly researched yet, we seek to explore possible abnormalities of the vessel wall in the macrovasculature of SSc patients by investigating the presence of progressive wall thickness of the vessels of the forearm.

Objectives

To investigate presence of progressive wall thickness between healthy controls and systemic sclerosis patients.

Secondary objectives

- To investigate the differences in vessel wall thickness of the brachial, radial, and ulnar artery, between the left and right upper extremities.
- To investigate possible differences between men and women.
- To investigate possible differences in lumen to IMT ratio in healthy controls and patients.

Study design

This project is an explorative subproject of an on-going study conducted in the UMCG on 'calcification as an early measure of systemic sclerosis' (CALC-SSc). The study will be conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). The study has been approved by the Medical Research Ethics Committee (MREC) or 'Medische Ethische Toetsing Commissie' (METC). Healthy participants aged between 40 and 75 years old were approached and invited for a visit to the vascular lab at the university medical center Groningen (UMCG). Healthy controls were matched on age and gender to SSc patients that were already included in the study.

All participants (including patients signed consent forms prior to the measurements. During their visit of approximately 2 hours several measurements are done (see Methods). A detailed description of the visit can be seen in appendix A. Furthermore, the CALC-SSc study collects other material and data including blood samples, blood pressure, finger blood pressure and nailfold capillary microscopy which will also be conducted during the visit (protocols in appendix B). All these procedures, laboratory and medical, were experienced first-hand (Appendix C). However, this data beyond the scope of this research project and thus not shown.

Materials and Methods

A comparison to healthy controls may give us a better understanding in the progression of the disease. We hypothesize that the IMT is increased in SSc compared to healthy controls. This research project may provide new insight in the involvement of the macrovasculature and in the early detection of SSc, which in turn allows possible early interventional actions.

Patient characteristics

Forty-two SSc patients (5 male, 37 female) and 12 healthy controls (3 male, 9 female) were studied. The median age (IQR) of SSc patients and healthy controls was 62 (54-68) and 58 (54-62) years respectively.

Forearm vasculature assessments

Vasculature assessments are performed using the VEVO®MD (Fujifilm, VisualSonics) ultra-high-frequency ultrasound machine. The brachial, ulnar, and radial artery of both arms were measured with a 48 MHz linear probe. Patients were laying down with their arms in supine position. Ultrasound gel was subsequently applied on the brachial, radial, and ulnar artery sites, followed by the ring- and index fingers. All the measurements were performed together with a trained vascular lab technician. Frozen longitudinal images are captured during the measurement and assessed on appearance and vessel wall thickness of the far wall (vessel wall most distant from the head of the probe) in millimeters using the available measuring software. IMT is defined as the mean IMT of the space from the leading edge of the lumen–intima interface to the leading most echogenic edge of the media–adventitia interface over a sufficient distance of the far wall[18]. Lumen diameter was defined as the distance between the near wall and far wall.

Severity Score

As the IMT cannot be properly measured with ultrasound in all vessel walls a rating scale to score each IMT or ultrasound image was adopted (0 = normal, 1 = abnormal, 2 = severe, 3 = stenosis). The IMT is scored 'normal' when within the standard deviation of the means of the control group (Table 1). 'Abnormal' is a score within the range of one and two standard deviations. When an IMT scores outside two standard deviations a score of 'severe' is given. If the ultrasound image is unmeasurable because of a stenosis and/or occlusion the vessel is severely affected by the disease, the score is ranked as 'stenosis'.

Statistical analysis

Data was expressed as numbers of subjects (percentages) mean ± sd, or median [interquartile range (IQR)] for categorical, normally, and non-normally distributed data, respectively. Normality is assessed using Q-Q plots and P-P plots and Levene's test of equality. Between-group differences are assessed using independent-samples t-test and Man-Whitney U tests. Differences between paired results are tested with a paired-samples t-test or Wilcoxon. For multiple group analysis ANOVA and K-independent samples tests will be implemented. Pearson's ranked test is used to determine correlations. Significance was set to $p < 0.05$. All data analysis was performed using SPSS v.28 (IBM, NY, USA).

Results

All images of the control group were measurable. In the SSc group one patient had a stenosis or occlusion of the right brachial site, nine patients for the right ulnar and seven patients of the left ulnar artery (table 2). Examples of ultrasound IMT measurements and different degrees of clarity are shown in figure 1(A-C).

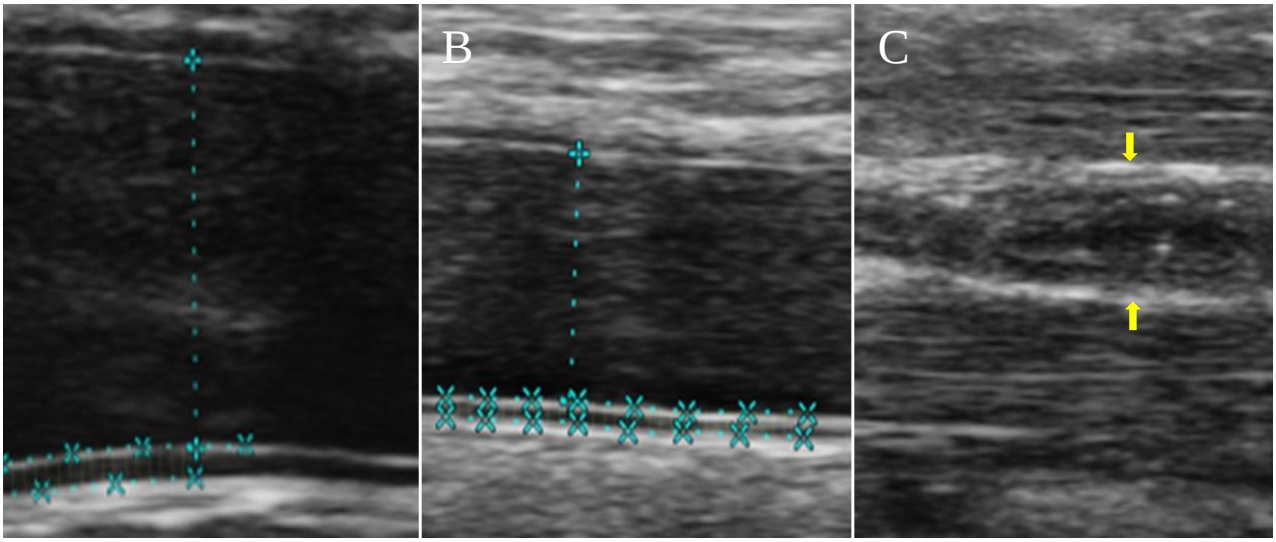

Figure 1: Sample ultrasound images (48 MHz) of A) clear, B) vague, and C) unmeasurable IMTs of patients. The yellow arrows in C indicate the vessel borders.

Severity score

The table 2 illustrates the severity scores and total percentages of the ultrasound images. Figure 2 provides another overview of the percentages of scores per vessel. It is apparent from this figure and table 2 that the ulnar artery of both arms has a larger extent of stenosis scores compared to the other vessels (left ulnar 21,4% and right ulnar 16,7%).

	Mean IMT (mm)	**Standard Deviation (mm)**
Right Brachial (RB)	0,31	0,057
Right Radial (RR)	0,23	0,049
Right Ulnar (RU)	0,30	0,096
Left Brachial (LB)	0,29	0,088
Left Radial (LR)	0,22	0,055
Left Ulnar (LU)	0,35	0,084

Table 1: Means and standard deviation of the IMT scores of the control group.

	Normal (%)	**Abnormal (%)**	**Severe (%)**	**Stenosis (%)**
Right Brachial	25 (59,5)	11 (26,2)	5 (11,9)	1 (2,4)
Right Radial	28 (66,7)	10 (23,8)	4 (9,5)	0 (0)
Right Ulnar	24 (57,1)	6 (14,3)	3 (7,1)	9 (21,4)
Left Brachial	32 (76,2)	8 (19)	2 (4,8)	0 (0)
Left Radial	27 (64,3)	12 (28,6)	3 (7,1)	0 (0)
Left Ulnar	26 (61,9)	6 (14,3)	3 (7,1)	7 (16,7)

Table 2: Ultrasound severity scores and percentages SSc patients (n=42). The IMT is scored 'normal' when within the standard deviation of the means of the control group (Table 1). 'Abnormal' is a score within the range of one and two standard deviations. When an IMT scores outside two standard deviations a score of 'severe' is given. If the ultrasound image is unmeasurable because of a stenosis and/or occlusion the vessel is severely affected by the disease, the score is ranked as 'stenosis'.

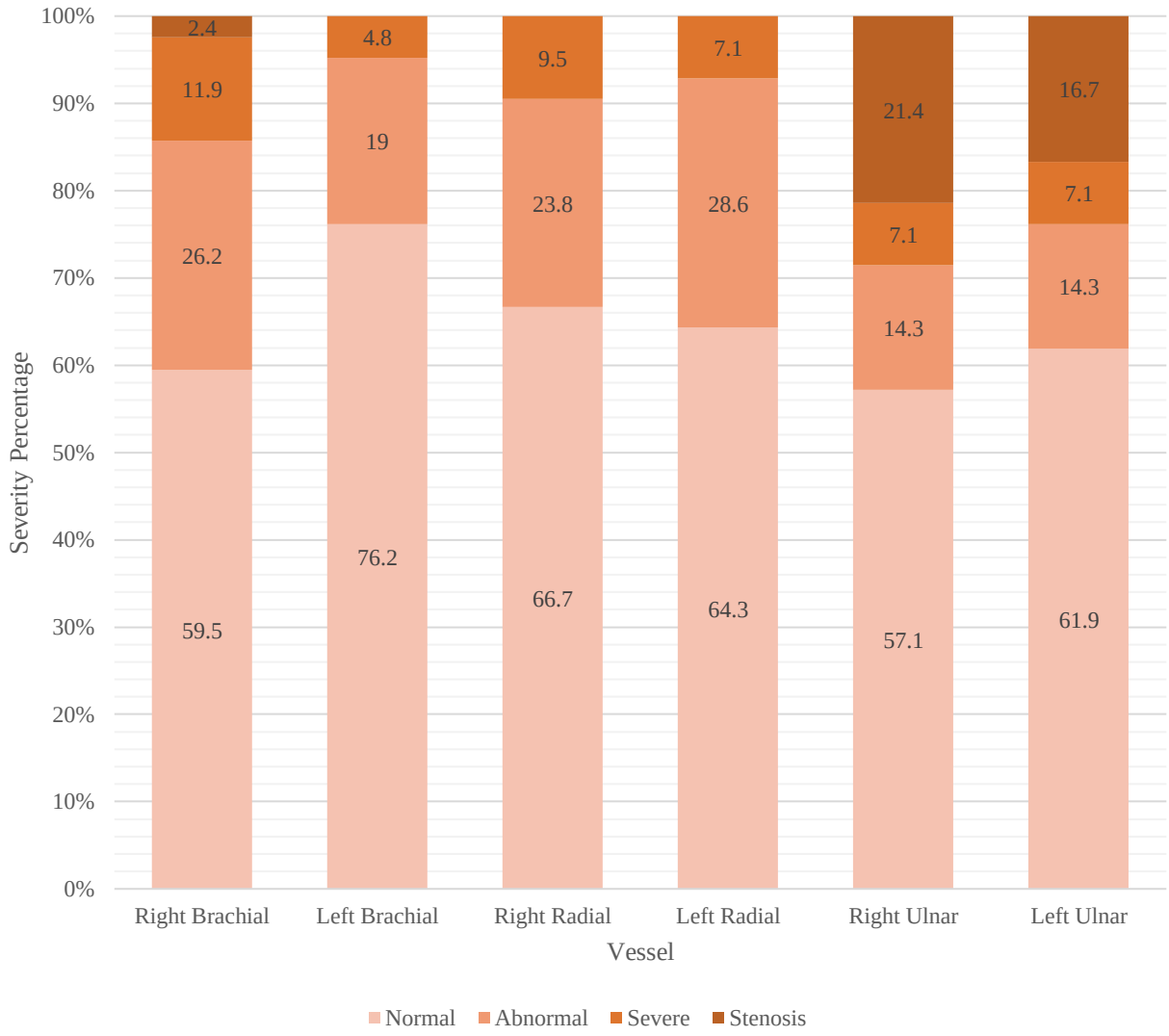

Figure 2: Severity score percentages of the SSc group.

IMT

One of the objectives of this research project aimed to elucidate possible differences in IMT using several comparisons. This was only possible in those patients with measurable IMT without stenosis (right brachial n=41, right radial n=42, right ulnar n=33, left brachial n=42, left radial n=42, left ulnar n=35). Comparing the vasculature between the right and left arm revealed no significant differences. When looking at gender differences in the patient group no difference was found. However, in the control group men had a significant higher IMT in the right brachial ($p = 0.013$), and the left radial artery ($p = 0.009$). In the control group the brachial artery IMT was increased, though not significantly. In patients, the radial and ulnar artery IMT were slightly increased, but here as well no significant difference was observed.

	Control (n =12)	SSc	*p*-value
Right Brachial (mm)	0.31 ± 0.057	0.28 ± 0.100 (n=41)	0.625
Right Radial (mm)	0.23 ± 0.049	0.25 ± 0.065 (n=42)	0.216
Right Ulnar (mm)	0.30 ± 0.096	0.33 ± 0.100 (n=33)	0.305
Left Brachial (mm)	0.29 ± 0.088	0.26 ± 0.059 (n=42)	0.852
Left Radial (mm)	0.22 ± 0.055	0.24 ± 0.052 (n=42)	0.181
Left Ulnar (mm)	0.35 ± 0.084	0.35 ± 0.088 (n=35)	0.781

Table 3: Mean IMTs ± SD of the control group and patient group in mm.

Lumen diameter

Interestingly, the diameter of the lumen was observed to possible help explain differences in IMT. Results show all arteries in the control were larger in lumen diameter (figure 3). Independent t-tests showed the radial and ulnar arteries diameter were significantly higher in the control group (RR (p=0.042), RU (p=0.001), LR (p=0.001) and LU (p<0.001)). Moreover, the diameter of the brachial arteries was also higher with borderline significance (RB (p=0.051), LB (p=0.076)). No significant differences were found between the left and right arm. In the patient group men had a significant higher diameter in the right brachial (p<0.001), right radial (p=0.003) and left brachial (p<0.001). In the control group this was only the case for the right ulnar (p=0.007) and left brachial (p=0.02).

IMT-lumen ratio

The IMT-lumen ratio might be of use as way to standardize IMT measurements, as larger vessels have a larger lumen, and possibly a larger (normal) IMT. The right ulnar and left radial ratios had significantly higher scores in patients (p=0.002 and p=0.028 respectively). Moreover, the left ulnar (p=0.062) and right radial (p=0.084) are elevated, though not significantly different (figure 4). No significant different was observed in the brachial arteries.

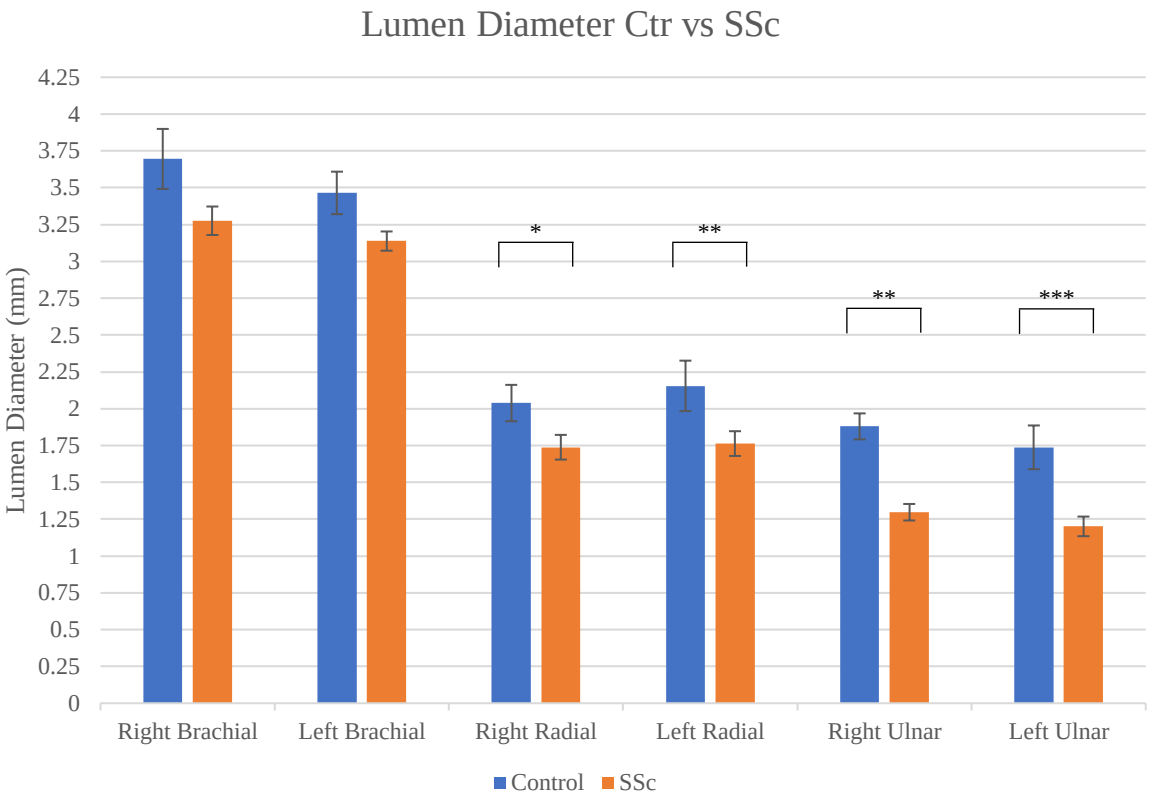

*Figure 3: Mean lumen diameter ± SD (Ctr (n=12) vs SSc (right brachial n=41, right radial n=42, right ulnar n=33, left brachial n=41, left radial n=42, left ulnar n=35)) *p<0.05 **p<0.01 ***p<0.001.*

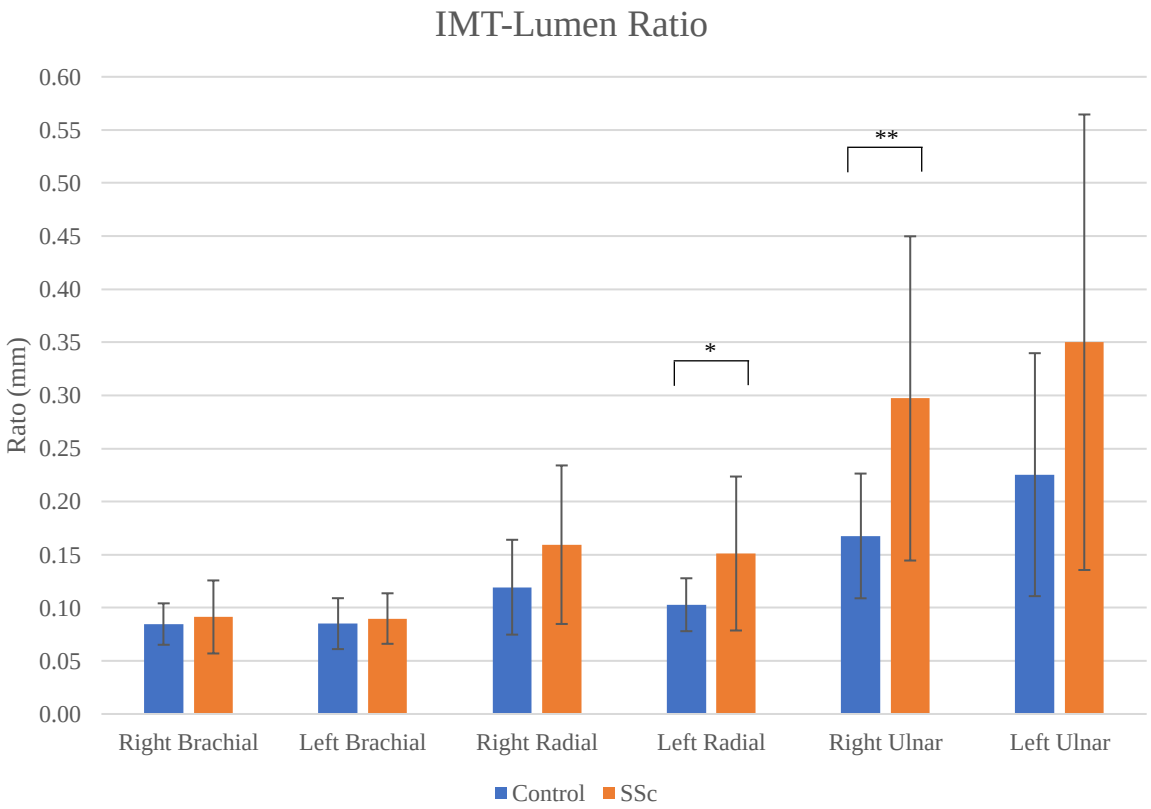

*Figure 4: IMT-Lumen diameter ratios. Control (n=12) vs SSc (Right Brachial n=41, Right Radial n=42, Right Ulnar n=33, Left Brachial n=41, Left Radial n=42, Left Ulnar n=35). *p<0.05 **p<0.01*

Discussion

Scientific literature surrounding vasculopathy in systemic sclerosis shows clear evidence that the microvasculature is affected but data on the involvement of the macrovasculature is scarce. This research project sought to determine the possible presence of progressive wall thickening in the macrovasculature of the forearm in systemic sclerosis. To explore said objective we looked at the IMT and the degree of severity of the brachial, radial and ulnar arteries. Also, gender, left/right, and vessel lumen diameter differences were examined. This research project did not observe any significant difference in IMT between the healthy control group and the SSc patients. However, this research did show an increased number of cases of stenosis and/or occlusion in the ulnar arteries. Furthermore, the radial and ulnar arteries indicated a smaller lumen diameter in patients compared to controls. In addition, the brachial arteries do not show any differences. Thus, this report indicates a non-influential role of the brachial arteries in SSc. Also, observing the results we can assume that the vasculature in the left and right arm are equally affected.

Severity score

The most obvious finding of this report is that the severity score of 'stenosis/occlusion' are higher in ulnar arteries (21,4% and 16,7%). Previous studies have indicated a role of ulnar artery in the development of SSc or as a severity marker of the vasculopathy (20,22). Moreover, a comparative study by Fairchild et al. (2021) indicated with radiography and ultrasound that ulnar artery occlusions are strongly associated with calcinosis (23). As mentioned before calcinosis is a known disease characteristic of SSc. Imaging assessments and physical examinations can be done to validate that these patients indeed are affected by calcinosis and appropriate countermeasures can be made. Interestingly, ulnar occlusion has been shown to be an important risk factor for the development of digital ulcers in patients with SSc, but this is outside the scope of the project (24).

IMT

Contradictory to our hypothesis we found no significant differences in IMT between the two groups. This may suggest that the IMT is not affected in the progression of SSc. These findings, however, are limited because only measurable IMT's were integrated in the analysis. It is highly likely that this data consists of less affected vessels or patients that are in an early stage of SSc, and that IMT probably is not the best indicator for vessel wall abnormalities in SSc. Examining the disease patterns and severity score might give more clarity to this discrepancy. It is also important to bear in mind that a relatively small patient sample size was used in the analysis. Furthermore, no clear consensus has been reached to where the intima-media-adventitia borders begin and start. In several ultrasound images it was somewhat unclear of where the adventitia begins, and the intima media ended since there was a vague gray area between the two borders. These possible sources of error could have affected the IMT values negatively and may give unrealistic results.

Follow-up study should include a second observer to rule out errors and provide more definitive results. Moreover, this research has thrown up many questions and opportunities for further investigation. Increasing the list of study variables to the analysis like, patient history, disease progression/stage or BMI can clarify inconsistencies in the data. Investigating the relation with different clinical parameters like, capillaroscopy and calcinosis can be possibilities for additional research as well. The relation with the severity of ischemia in the fingers can also be

investigated by measuring the finger blood pressures. In addition, further research should focus on better determining the IMT borders because that was one of the main difficulties during this research. Investigating more accurate visualization equipment or techniques can be a research possibility because current machines do not have the necessary specifications yet.

Conclusion

Despite its exploratory nature, this research project's outcomes support the notion that the macrovasculature (radial artery and ulnar artery) are involved in the progression of SSc and can provide support for future research and interventional treatment options regarding SSc.

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Appendices

A. Subject visit step-by-step plan

Procedure healthy controls visit	By:
1. Invite individual for a visit via e-mail (include information and informed consent letter).	**Tobias**
2. Discuss availability vascular lab and individual with *Saskia.*	**Tobias**
3. Schedule an appointment for a visit by e-mail. Pass on the time and location of arrival to the volunteer (Vascular lab UMCG).	**Tobias**
4. Preparation forms/necessities for appointment: • Information letter • Informed consent letter • Blood collection forms (Dummy nr.) • Blood tubes (2x 10cc Serum, 3x 10cc EDTA, 2x 2,5cc) (Dummy nr.) • VVV voucher	**Tobias**
5. Pick up volunteer at the vascular lab	**Tobias**
6. Go through the information letter and have the declaration of consent signed by the volunteer. Answer any questions from the volunteer.	**Tobias/Bart**
7. Make a copy of the signed consent form to give to volunteer.	**Tobias/Bart**
8. Nailfold capillaroscopy measurement.	**Saskia**
9. Finger blood pressure measurement.	**Saskia/Tobias**
10. Bilateral arm blood pressure measurement.	**Saskia/Tobias**
11. Ultrasound measurement brachial, radial, ulnar artery and index- and ringer fingers.	**Saskia**
12. Supervise volunteer to blood collection at the blood clinic.	**Bart/Tobias**
13. Blood collection.	**Blood Clinic**
14. Transport blood + forms to the chemical lab.	**Bart/Tobias**
15. Supervise volunteer to the vascular lab.	**Bart/Tobias**
16. Take medical history of volunteer.	**Bart**
17. Give VVV voucher and, if possible, travel allowance claim form to volunteer. Answer any remaining questions.	**Tobias**
18. If necessary, supervise volunteer to the exit.	**Tobias**
19. Analyze ultrasound images and clips. Export to USB.	**Tobias**

B. Vascular lab and blood sampling procedures

Nailfold capillary microscopy is performed using a high-resolution camera as described by van Roon et al (2016). Every finger (excluding the little fingers) is assessed for the following characteristics: widened capillaries, giant capillaries, capillary density, and total loop width are assessed. Images are made and evaluated after the measurements. From these images and SSc patterns ('Early', 'Active' or 'Late'), defined by Cutolo et al. (2004), summarized in the "microangiopathy evolution score" (MES) and DU risk score (measured by Capillaroscopic Skin Ulcer Risk Index (CSURI) (26). Prognostic Index for Digital Lesions (PILD) are assessed (27). Immersion oil is applied before every measurement to increase skin transparency.

Finger blood pressure is measured using small blood pressure cuffs around each finger as well as infrared sensors to observe blood flow. Moreover, arm blood pressure is measured on both arms simultaneously to ensure significant results and comparison with the finger blood pressure.

Blood sampling

Six tubes of blood will be collected during the blood sampling. Blood for plasma will be collected in 1x 10 cc tube and 1x 10 cc tube will be collected for serum storage. 3x 10 cc EDTA tubes will be collected for PBMCs isolation. 2x 2.5cc tube of blood is collected for RNA interferon signature (Paxgene). Various protein levels will be measured by commercially available ELISAs, or routine procedures available at the UMCG clinical chemical lab. All the discussed laboratory procedures are experienced first-hand.

From whole blood, peripheral blood mononuclear cells (PBMCs) will be isolated and stored at -80 °C in liquid nitrogen for determination of angiogenic T cells and Tie2 positive monocytes (see appendix C). Frequencies of CD3+, CD4+ and CD8+ angiogenic T cell populations will be analyzed in thawed PBMCs by flow cytometry on the LSR II (BD Bioscience) using antibodies against surface markers CD3-PerCP, CD8-AF700, CD31-PB and CXCR4-BV605 (CD184) (all from Biolegend). Compensation will be done using isotype controls. This data is not included for the reason mentioned above.

C. PBMC isolation protocol

Materials

Lymphoprep (Sterile, at room temperature)
RPMI+gentamycine(500ml+3ml, at room temperature)
RPMI+20%DMSO (16 ml+4ml,on ice)
RPMI+20%FCS (16 ml+4ml,on ice)

Methods

1. Peripheral blood in 10ml lithium heparin tubes (green).
2. Add 4ml Lymphoprep to each 15ml tube.
3. Add RPMI (with gentamicin) to blood: 1: 1 , pour blood in 50ml tube.
4. Carefully add 8 ml of diluted blood to Lymphoprep.
5. Centrifuge tubes (sufficient support tubes at the bottom with program6 (2400rpm, Break 0, 20min).
6. Remove supernatant down to 0.5 cm above white ring in circular movement, collect white part to a new 15ml tube, add RPMI medium to wash.
7. Centrifuge / washing with program 7 (1800 rpm, brake5, 10 min).
8. Remove supernatant, resuspend with 10 ml RPMI and pool all the pellets together and centrifuge with program 4 (1200 rpm, brake 5, 10 min).
9. Remove supernatant and resuspend cell pellet with 1ml RPMI+20%FCS, count (20ml counting suspension+40µl cell suspension).
10. Divide cell suspension over freezing tubes (on ice), add RPMI+20%DMSO and RPMI+20%FCS, ended with 1×10^7 cell+10%FCS+10%DMSO.
12. Place tubes in isopropanol freezing container (room temperature; use up to 5 times) and put it in -80℃ freezer.
13. After 24 hours of freezing, put tubes in nitrogen container.