Title

Complement proteins and arterial calcification in middle aged women: Cross-sectional effect of cardiovascular fat. The SWAN Cardiovascular Fat Ancillary Study.

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ASSOCIATIONS BETWEEN COMPLEMENT PROTEINS AND ARTERIAL CALCIFICATION IN MID-LIFE WOMEN: ROLE OF CARDIOVASCULAR FAT, THE STUDY OF WOMEN'S HEALTH ACROSS THE NATION (SWAN)

ABSTRACT:

The risk of cardiovascular disease (CVD) increases in women after menopause along with levels of complement protein C3. Recent data has shown higher cardiovascular fat in postmenopausal women. Increasing evidence suggest this fat depots are a source of cytokines and various inflammatory markers. Both complement protein C3 and cardiovascular fat are associated with increased risk of CVD. The association between these factors needs to be evaluated in women at midlife.

Hypothesis:

Circulating complement protein levels in women at midlife are positively associated with arterial calcification, and this association can be explained by higher volumes of cardiovascular fat.

Methods:

Pilot data from the SWAN were used. C3 and C4 were measured by immunoturbidimetric assay. EBCT scans were used to measure the arterial calcification (aortic-AC and coronary-CAC) using Agatston scores and the volumes of fat around the heart (total heart adipose tissue-TAT) and the descending thoracic aorta (perivascular adipose tissue-PVAT). Arterial calcification and fat volumes were log transformed. Tobit regression was used for statistical analyses.

Results:

A total of 100 women (50% late peri/postmenopausal; 73% Caucasian) were included. In models adjusted for age, race, menopausal status, and LDL-C, C3 was significantly associated with both CAC (β(SE)=0.43(0.17),p=0.012) and AC (0.59(0.28),p=0.036) per 1 standard deviation increase of C3 (SD=33.28 mg/dl). Additional adjustment for either TAT or PVAT nullified the association of C3 with both CAC and AC. Association between C3 and AC was more pronounced at higher volumes of TAT, independent of potential covariates (p=0.036). C4 was not associated with any of the calcification measures.

Conclusions:

Higher levels of C3 were significantly associated with greater CAC and AC scores in women at midlife. These associations were explained by volumes of TAT and PVAT. Our findings extend support to the outside-in theory of atherosclerosis and suggest TAT as a potential source of circulating C3. Similar results are reported in unhealthy populations. The public health significance lies in the fact that by extending these findings to the general population, we have potentially found a non-invasive biomarker that could be useful in early diagnosis of subclinical atherosclerosis. These findings need to be replicated in larger samples.

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1.0 iNTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States (1). The risk of CVD in women increases significantly after the fifth decade of life, which is around the time of the menopausal transition (2). Interestingly, circulating complement proteins such as C3 have been shown to increase in women during this time period (3, 4) and to be associated with menopausal status (5). These findings suggest a potential role of complement proteins in explaining the higher risk of CVD after menopause. Although the exact mechanism of the associations between complement proteins and atherosclerosis is not clear, evidence shows that complement proteins can be activated within the plaque (6, 7). Additionally, previous studies reported significant associations between C3, C4 and subclinical measures of atherosclerosis such as coronary calcification and carotid intima medial thickness (8-11). However, these studies were not specific to women transitioning through menopause, and were mainly conducted in unhealthy populations suffering from autoimmune diseases.

Women transitioning through menopause undergo changes in their body fat distribution including an increase in abdominal adiposity (12-14). Importantly, recent findings from the

Study of Women's Health Across the Nation (SWAN) Cardiovascular Fat ancillary study showed that postmenopausal women tend to have higher volumes of fat around the heart (15). Fat around the heart (epicardial and paracardial) and vasculature (descending thoracic aorta) have been shown to be associated with a higher risk of CVD and subclinical measures of atherosclerosis (16-20, 11). This supports the "outside-in" theory of atherosclerosis, which postulates that inflammation is initiated within the surrounding perivascular adipose and progresses through the adventitia into the intima (21, 22). Interestingly, studies on mouse models showed that increased C3 and C4 deposition were associated with atherosclerosis and vascular stiffness. These complement proteins were found to be bound to the adventitial and medial fibers including collagen and elastin at early time points prior to luminal lesion development thus suggesting the perivascular aortic fat as a potential source (6). In fact, C3 and C4 proteins are strongly associated with abdominal adiposity and visceral adipose tissue in human related studies (23-25). Taken together, as women transition through menopause, fat around the heart and vasculature may increase and become a potential source of local complement proteins C3, which is known to be related to CVD risk and subclinical atherosclerotic measures (23, 26).

For the present study we aim to evaluate the association between circulating complement proteins (C3 and C4) and arterial calcification in the coronary arteries and aorta (measures of subclinical atherosclerosis, CAC and AC, respectively), and to evaluate if this association varies by the volume and location of the cardiovascular fat.

2.0 METHODS

Study of Women's Health Across the Nation (SWAN) is an ongoing, multi-site, longitudinal study of women examining the physiological and psychological changes during their transition through the middle years. The study design has been previously published (**27**). Between 1996 and 1997, 3302 participants were recruited from 7 different sites across the country (Boston, MA; Oakland, CA; Los Angeles, CA; Detroit, MI; Chicago, IL; Pittsburgh, PA & Newark, NJ). The eligibility criteria for the study were (1) An intact uterus and at least 1 ovary; (2) Not pregnant or breast feeding; (3) At least 1 menstrual period within the past 3 months; (4 No hormone therapy use within the past 3 months.

The participants of the current study were part of an ancillary study to the SWAN study at the Pittsburgh site. A random pilot sample of 100 participants was obtained based on the availability of the serum samples and menopausal status (50% pre- or early peri-menopausal and 50% late peri- or post-menopausal). The participants provided written informed consent prior to enrollment and research protocols were approved by the University of Pittsburgh institutional review board (IRB).

2.1 STUDY MEASURES

2.1.1 Arterial Calcification

Coronary artery calcification (CAC) and aortic calcification (AC) were measured using electron beam computed tomography (EBCT) scans in 3 passes. The first pass marked the anatomical landmarks for the coronary and aortic scans. The second pass showed the coronary

arteries and were obtained at maximal breath hold using electrocardiographic triggering to obtain the 100-ms exposure in the same phase of cardiac cycle of R-R interval (60%). The third pass captured the aortic artery from the aortic arch to the aortic bifurcation. The scans were saved on an optical disc. To assess CAC, 30-40 contiguous scans of 3-mm were obtained from the level of root of aorta to the apex of the heart at maximal breath holding. To assess aortic calcification, 6-mm images were obtained picturing the arch of the aorta to bifurcation of the iliac vessels with a 300-ms exposure. AC and CAC were assessed using a DICOM workstation equipped with AcuImage, Inc software (South San Francisco, CA) at the University of Pittsburgh using the Agatston scoring technique (28). Calcification was determined as present if three contiguous pixels showed > 130 Hounsfield units (HU). CAC was determined as the sum of Agatston scores of the 4 major coronary arteries (29).

2.1.2 Blood assay

Blood samples were collected in the morning after fasting overnight (8-12 hours). The blood collection was scheduled on days 2-5 of a regular menstrual cycle. In cases when a scheduled sample could not be obtained (due to less regular menstrual cycles), a random fasting sample was collected within 3 months of the scheduled annual visit. After sample collection, the blood was maintained at 4° C and separated and frozen at -80 ° C. The sample was then transported to the medical research laboratories on dry ice for analysis. High density lipoprotein cholesterol (HDL-C) was measured using heparin-2M manganese chloride while triglycerides and total cholesterol were measured by enzymatic methods using a Hitachi 747 analyzer (30-32). Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation (33), after excluding triglyceride value >=400 mg/dl. Insulin resistance was measured as homeostasis

model assessment insulin resistance index (HOMA-IR) using fasting insulin and glucose levels (34). C3 and C4 were assessed from frozen serum samples using commercial immunoturbidimetric assay kits (Tina-quant C3 cassette and a Tina-quant C4 cassette) that were run on the Integra 800.

2.1.3 Total heart and perivascular adipose tissue volumes

Volumes of total heart adipose tissue (TAT) and perivascular adipose tissue (PVAT) of the descending thoracic aorta were quantified using existing EBCT scans previously obtained to measure arterial calcification (Fig 1). TAT was defined as the fat around the heart and PVAT was defined as the fat around the descending thoracic aorta.

TAT volume was quantified at the Biomedical lab at the Harbor UCLA Medical center. For TAT volume (cm^3), slices within 15 mm above and 30 mm below the superior extent of the left main coronary artery were included. The anterior border was defined by the chest wall and the posterior border by aorta and bronchus. This region was chosen as it also consists of the epicardial fat around the proximal coronary arteries including left anterior descending, left main coronary artery, right coronary artery and circumflex arteries. The protocol to quantify TAT showed excellent reproducibility with a between-readers Spearman correlation coefficient of 0.99 and within-reader Spearman coefficients of at least 0.97 (35).

PVAT volume was quantified as previously described (11) using the existing scans originally obtained to quantify AC. The scans were re-read at the University of Pittsburgh using the software Slice-O-matic (Tomovision, Montreal, Canada) by one local reader. The posterior border for PVAT was defined as the anterior portion of the spinal foramen while the anterior and lateral borders were defined by the left bronchus, esophagus and crus of diaphragm. The

pulmonary bifurcation was used as the proximal boundary of the descending aorta while the distal boundary was marked by the initial image of the first lumbar vertebrae. The adipose tissue volumes for both TAT and PVAT were quantified by using Hounsfield Unit (HU) attenuation values for fat (-190HU to -30 HU) (36). Similar to TAT, the PVAT measures have shown excellent reproducibility statistics with a Spearman correlation coefficient for both between reader and within reader of 0.99 (11).

2.1.4 Study covariates

Age was calculated using the birth date reported upon screening. Race/ethnicity was self-reported. The body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Heart medication and smoking (current smoker or non-smoker) were self-reported.

Menopausal status was determined based on reports of the frequency, and regularity of menstrual bleeding and use of hormone therapy and were classified into: (1) Premenopausal - women experienced no changes in cycle intervals, (2) Early peri-menopausal - women had at least 1 bleeding cycle in the last 3 months with a perceived change in the cycle interval, (3) Late peri-menopausal - had 3 consecutive months without a menstrual cycles, (4) Postmenopausal - had 12 consecutive months without a menstrual cycle. Due to small sample size, the premenopausal and early peri-menopausal groups were combined together as premenopausal, while the late-peri and post-menopausal groups were combined together as postmenopausal.

2.2 STATISTICAL ANALYSIS

Independent (C3, C4) and dependent variables (CAC and AC) as well as covariates were examined for distribution and outliers. The distributions of CAC, AC, TAT and PVAT were skewed and therefore log transformation was applied to all these measures.

2.2.1 Tobit regression

Since AC and CAC tend to have lower bound scores (~0), we analyzed the data using Tobit regression (36). Tobit regression modelling is a type of censored modelling which estimates the linear relationships between variables which tend to have a right or left censoring (clustering). Interpretation of coefficients from Tobit regression is similar to that from linear regression. For the current analyses, marginal effects of the independent variable on the outcome (observed and censored) were presented. Separate Tobit regression models for each of the arterial calcification measures and each complement proteins were fitted. Models were adjusted for potential covariates based on results from univariate analysis. Only the covariates found to be significantly associated with arterial calcification with a p-value of <0.1 were considered in multivariable analyses. Since HDL-C and triglyceride levels were highly correlated with C3 and C4 levels (>0.4) these variables were not included in multivariable analyses to reduce collinearity.The effect sizes were calculated per 1 SD of the complement protein levels. Statistical analyses were performed using STATA 13 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP) with two sided test and significance level of 0.05.

3.0 RESULTS

The characteristics of the population are summarized in Table 1. The mean age of the women in the study was 50.48 years with 73% of them being Caucasians. The population tended to be overweight with a mean BMI of 28.66 kg/m^2 and the majority being nonsmokers (84%). The study population had high LDL-C values with a median of 120 mg/dl.

In univariate analysis (Table 2), higher levels of CAC were significantly associated with higher BMI (p=0.001), LDL-C (p=0.022), triglycerides (p=0.001), TAT (p=0.001), PVAT (p=0.001) and complement protein C3 (p=0.001). Similar significant associations were seen between AC and BMI, triglyceride levels, adipose tissue volumes and C3. Both CAC and AC were inversely and significantly associated with HDL-C levels (p<0.002). No significant association was found between C4 and CAC or AC.

The association between complement protein C3 and CAC (Table 3) remained significant after adjusting for age, race and menopausal status (p=0.001) (Model 1). The association remained significant even after additional adjustment for LDL-C (p=0.012) (Model 2). However, on additional adjustment for either TAT or PVAT, the association between C3 and CAC disappeared. Similar results were seen between C3 and AC. No statistically significant associations were found between C4 and the calcification measures.

Next, we assessed the interaction between complement proteins and cardiovascular adipose tissue volumes in relation to the arterial calcification measures. A significant interaction was found between C3 and TAT (p=0.028) in relation to AC. The interaction remained significant after adjusting for age, race, menopausal status and LDL-C (p=0.036) (Fig 2). The association between

C3 and AC was more pronounced at greater volumes of TAT. No significant interaction was found between C3 and TAT in relation to CAC scores.

4.0 DISCUSSION

Our study showed that higher levels of circulating complement proteins were significantly associated with greater levels of CAC and AC in healthy midlife women. These associations were explained by the volumes of TAT and PVAT. Furthermore, the association between AC and C3 was more pronounced at greater volumes of TAT. These findings suggest a potential link between complement proteins and cardiovascular fat in relation to subclinical atherosclerosis levels in women at midlife.

Complement proteins – especially C3, have been vastly studied in relation to CVD and subclinical atherosclerosis. Higher levels of circulating C3 have been shown to be associated with arterial calcification in different at-CVD risk populations such patients suffering from various autoimmune diseases like SLE and psoriasis, and elderly patients(>70 years) (9, 10, 23, 26). The findings from our study are consistent with these results and extend it to healthy women at midlife.

Complement proteins are plasma proteins that are part of the body's innate immune system. Through a cascade of activations, they amplify the inflammatory response (37). The role of complement proteins in the process of atherosclerosis has become increasingly evident (38, 39). Studies on C3 deficient mice models have demonstrated that an intact complement system is required for the development of atherosclerosis (40). In human related studies, this association

has been widely studied in relation to autoimmune conditions such as SLE and psoriasis. In addition, follow up studies in women with pre-existing CAD have shown that high levels of circulating C3 could predict the complications of atherosclerosis (41). The results of our study are consistent with these findings and support the evaluation of circulating complement levels in association with CVD in a broader population of healthy women at midlife.

Recent studies have advocated for an inflammatory pathway in the process of atherosclerosis (42). However, the exact role of complement activation in development of atherosclerosis through such a pathway is not fully understood. Currently, it is believed that the complement activation is responsible for recruitment of other inflammatory mediators and thus increases the number of inflammatory cells at the targeted site (7) resulting in the formation of the membrane attack complex (MAC)(43), which results in damage to microvasculature through the production of reactive oxygen species (ROS)(44). This suggested mechanism, has been supported by reporting intense deposits of complement proteins in the coronary arteries (44). Additionally, complement protein C3 and the MAC have been found within the atherosclerotic plaques (45, 46).

Similar to complement proteins, adiposity is a well-established risk factor to CVD and subclinical CVD (47). In particular, visceral adipose tissue depots have been known to be associated with various cardiometabolic risk factors and higher risk of CVD (48). Adipose tissue depots such as epicardial (17-19) and pericardial (49) fat have been shown to be positively associated with CAC. Similar associations were noted with both the fat around the coronary blood vessels (50) and the aorta (51) in relation to atherosclerosis. This suggests a plausible role of cardiovascular fat depots in the atherosclerotic process. One of the theories put forth in support of this

relationship is the “outside-in” theory of atherosclerosis which suggests that the process of atherosclerosis is initiated within the surrounding PVAT and adventitial layers progresses towards the inner layers of the blood vessel including the media and intimal layers (21). Specifically, fat around the adventitia is believed to release pro-inflammatory adipokines (cytokines from the fat) which stimulate various inflammatory cells. Furthermore, this theory has been supported by the presence of various inflammatory cells within the layers of the vasculature (52, 6). The lack of a fascial barrier prevents any potential block to the migration of these inflammatory products from the fat depots into the vessel wall (6). Thus taken together, the presence of fat depots in such close proximity to the vessel wall with no fascial separation, and the increased circulating levels of complement proteins in this population suggest a possible link between these factors in the atherosclerotic process.

The results of our study not only suggest an association between complement proteins and arterial calcification in healthy midlife women, but also propose a plausible role of the cardiovascular depots in explaining this association. Previous studies provide evidence for a potential link between adipose tissue and complement proteins (23). Complement protein expression has been detected in visceral (omental) adipose tissue in obese individuals (25). Thus taken together, the findings of our research extend support to the “outside-in” theory and suggest TAT as a potential source of these complement proteins. To the best of our knowledge, such an association in healthy women at midlife transitioning through menopause has not been assessed.

However, the current findings should be viewed in the context of several limitations as follows: (a) the small sample size; (b) the cross sectional design , which limited our ability to assess

temporality; (c) our study population were predominantly Caucasian and thus cannot be generalized to other racial/ethnic groups. Despite these limitations, our study is the first to evaluate associations between complement proteins and calcification in healthy women at midlife and to assess the potential role of cardiovascular fat in explaining this association. Additionally, our findings would help in enhancing our understanding of the possible mechanisms of atherosclerosis. Future studies should replicate our study, however in a larger multi-racial/ethnic population and followed up over time to look for risk and occurrence of CVD. In addition, studies exploring the other circulating and deposited complement protein levels within the plaques and adipose tissue volumes shall help explain specific complement activation pathways and mechanisms.

5.0 CONCLUSIONS

In conclusion, circulating complement protein C3 levels are significantly associated with arterial calcification measures in women at midlife. These associations are explained by volumes of fat around the heart and thoracic aorta. Higher volumes of TAT significantly amplify the association between C3 and AC, suggesting this fat depot as a potential source of complement proteins and extend support to the “outside-in” theory. By extending the findings of our study to the general population, C3 could be used as a non-invasive biomarker for the early detection of subclinical atherosclerosis in this population. Considering that women are at a higher risk of CVD after the 5^{th} decade of life (2), early detection of arterial calcification without the use of EBCT scans in this population would not only be helpful in developing appropriate preventive strategies but prove

to be cost effective. It is important to replicate our study in larger study of multi/ethnic women at midlife over time.

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6.2 DISCLOSURES

Dr. Matthew Budoff is a consultant to GE. Other authors have no disclosures.

Table 1: Characteristics of the study population

Characteristic	**Total (N=100)**
Age (years), Mean (SD)	50.48(2.63)
Race: N(%) African Americans Caucasians	 27(27) 73(73)
Body mass index (kg/m2), Mean (SD)	28.66(5.92)
Smoking: N(%) Current smoker Non smoker	 15(15.15) 84(84.85)
Heart medication: N(%) Yes	 03(03)

No	97(97)
HDL-C(mg/dl),Median(Q1, Q3)	57(48,68)
LDL-C(mg/dl), Median(Q1, Q3)	120(105,145)
Triglycerides(mg/dl), Median(Q1, Q3)	101(79,144)
HOMA-IR,Median(Q1, Q3)	1.83(1.48,2.75)
Total Heart Adipose Tissue (cm3), Median(Q1, Q3)	43.47(35.12,66.59)
Perivascular Adipose Tissue (cm3), Median(Q1, Q3)	27.31(22.73,36.39)
C3 (mg/dl), Mean (SD)	162.3(33.28)
C4 (mg/dl),Mean (SD)	31.93(8.78)
Aortic calcification (AC) (Agatston score),Median(Q1, Q3)	2.80(0,4.67)
Presence of AC (AC>0): N(%) No Yes	 33(33) 67(67)
Coronary calcification (CAC) (Agatston score),Median(Q1, Q3)	0(0,2.29)
Presence of CAC (CAC>0): N(%) No Yes	 52(52) 48(48)

Table 2: Univariate analysis between study variables and arterial calcification measures

	CAC [a]		**AC** [a]	
	β(SE)	**p-value**	**β(SE)**	**p-value**
Age (years)	0.10(0.06)	0.086	0.08(0.09)	0.423
Race :				
African Americans	0.52(0.34)	0.127	0.47(0.55)	0.401
Caucasians	--	--	--	--
Body mass index (kg/m2)	**0.14(0.02)**	**0.001**	**0.19(0.03)**	**0.001**
Smoking :				
Current smoker	-0.28(0.45)	0.533	0.57(0.69)	0.409
Non smoker	--	--	--	--
Menopausal status:				
Post-menopausal	0.41(0.31)	0.196	0.25(0.50)	0.612
Pre-menopausal	--	--	--	--
Heart medication :				
Yes	0.86(0.87)	0.322	0.24(1.46)	0.869
No	--	--	--	--
HDL-C(mg/dl)	**-0.04(0.01)**	**0.001**	**-0.05(0.17)**	**0.002**
LDL-C(mg/dl)	**0.01(0.004)**	**0.022**	0.01(0.007)	0.062
Triglycerides(mg/dl)[a]	**1.14(0.29)**	**0.001**	**2.03(0.44)**	**0.001**
Homa-IR[a]	**1.26(0.19)**	**0.001**	**1.74(0.34)**	**0.001**
Total Heart Adipose Tissue (cm3)[a]	**1.57(0.32)**	**0.001**	**1.84(0.53)**	**0.001**
Perivascular Adipose Tissue (cm3)[b]	**2.05(0.40)**	**0.001**	**2.34(0.67)**	**0.001**
C3 (mg/dl) [c,d]	**0.59(0.15)**	**0.001**	**0.72(0.24)**	**0.002**
C4 (mg/dl) [c]	0.21(0.16)	0.180	0.13(0.25)	0.599

[a] log(CAC+1), log(AC+1); [b] log transformed;

[c] β per standard deviation (SD) ; SD of C3 = 33.28 mg/dl ; SD of C4= 8.77 mg/dl

[d] One SD increase in C3 is associated with 0.59 unit increase in the predicted value of CAC and a 0.72 unit increase in the predicted value of AC.

Table 3: Multivariable analysis between complement proteins and arterial calcification measures

	CAC[a]		AC[a]	
	C3			
	β(SE) [b]	**p-value**	**β(SE)** [b]	**p-value**

Unadjusted	**0.59(0.15)**	**0.001**	**0.72(0.24)**	**0.002**
Model 1[c,e]	**0.53(0.16)**	**0.001**	**0.75(0.27)**	**0.005**
Model 2[c]	**0.43(0.17)**	**0.012**	**0.59(0.28)**	**0.036**
Model 2 + TAT[d]	0.22(0.17)	0.191	0.12(0.31)	0.698
Model 2 + PVAT[d]	0.15(0.16)	0.367	0.31(0.29)	0.294
C4				
	β(SE)	**p-value**	**β(SE)**	**p-value**
Unadjusted	0.21(0.16)	0.180	0.13(0.25)	0.599
Model 1[c]	0.19(0.16)	0.213	0.11(0.26)	0.684
Model 2[c]	0.17(0.16)	0.301	0.04(0.26)	0.873
Model 2 + TAT[d]	0.10(0.15)	0.480	-0.24(0.26)	0.373
Model 2 + PVAT[d]	0.06(0.14)	0.679	-0.07(0.25)	0.769

[a] log(CAC+1), log(AC+1); [b] β per standard deviation (SD) - SD of C3= 33.28 mg/dl, SD of C4= 8.77 mg/dl; [c] Model 1- adjusted for age, race and menopausal status; Model 2 – Model 1+ LDL-c

[d] log transformed; [e] One SD increase in C3 is associated with 0.53 unit increase in the predicted value of CAC and a 0.75 unit increase in the predicted value of AC when adjusted for age, race and menopausal status

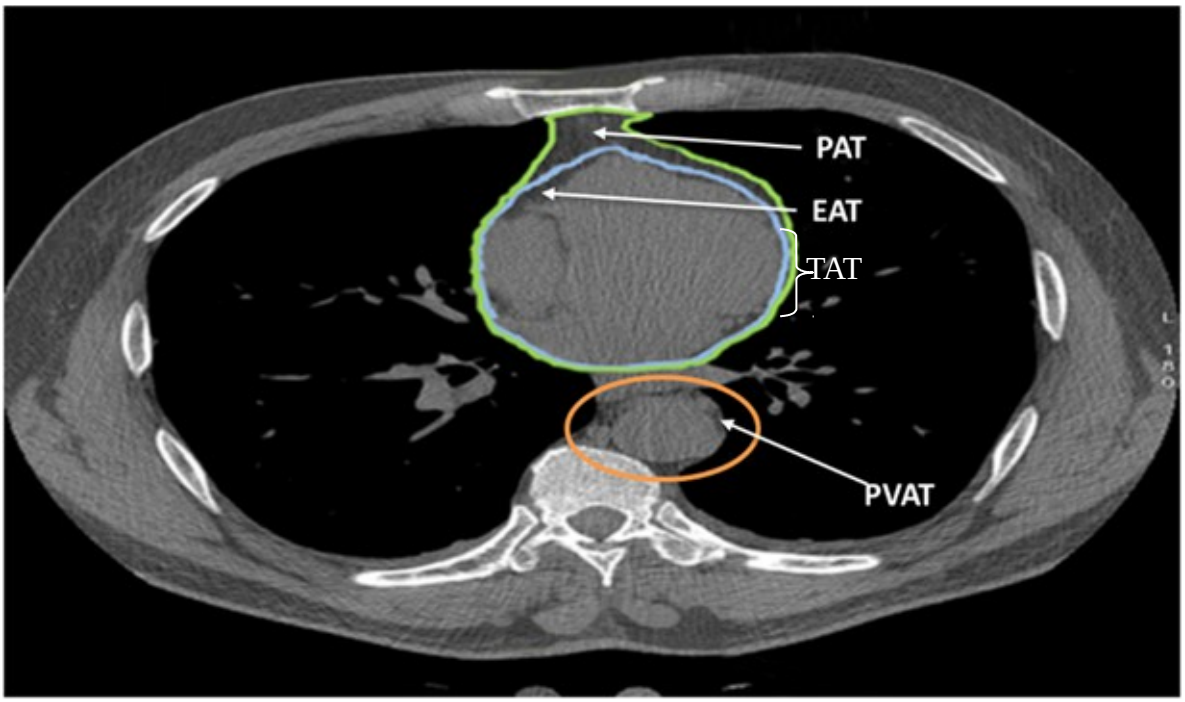

Figure 1: The total heart adipose tissue (TAT) volumes were quantified as the sum of epicardial (EAT) and paracardial adipose tissue (PAT). Perivascular adipose tissue (PVAT) was assessed as the fat around the descending aorta.

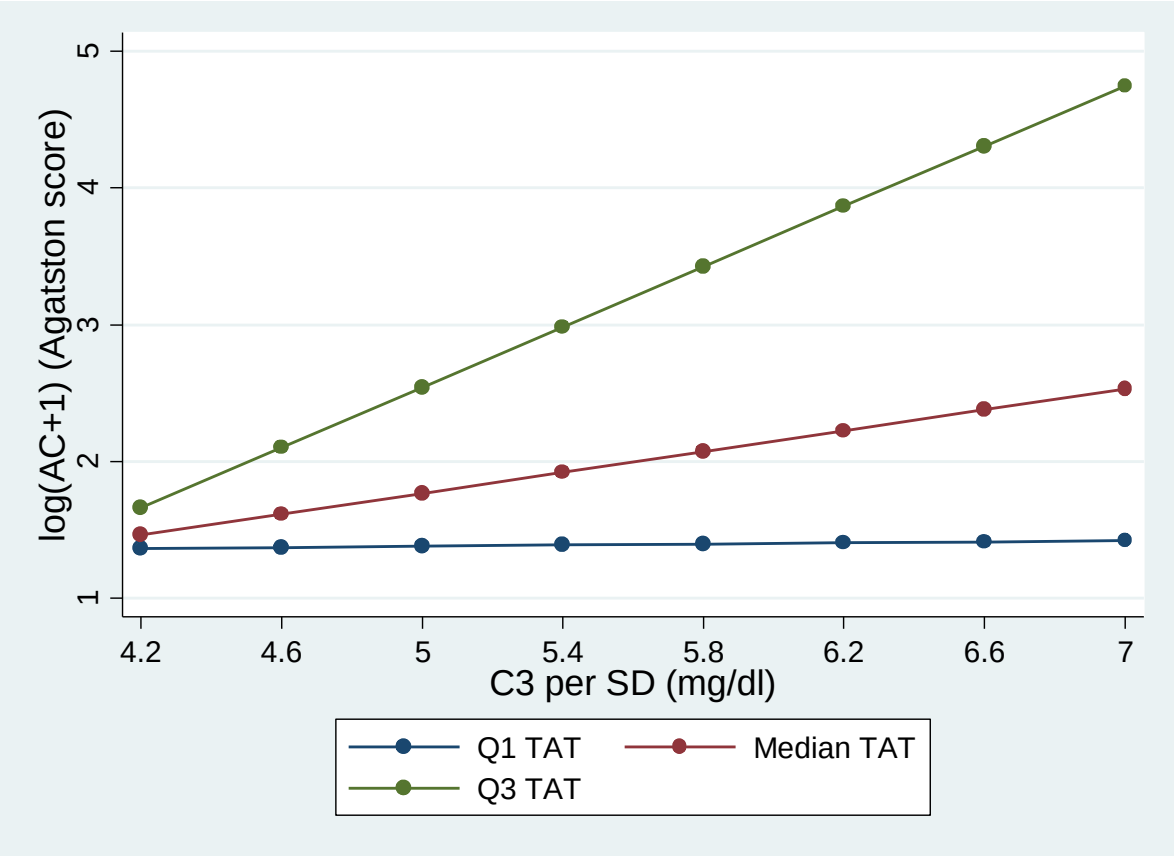

Figure 2: Interaction between C3 and TAT in relation to AC (adjusted for age, race, menopausal status and LDL-C)

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