



Published in final edited form as:

Exp Physiol. 2018 November ; 103(11): 1560–1570. doi:10.1113/EP087116.

Type 2 diabetes and older age contribute to elevated plasma microparticle concentrations independent of chronic stroke

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Abstract

Elevated circulating concentration of endothelial microparticles (MPs) may provide an index of the extent and nature of cellular damage in chronic stroke. The purpose of this study was to determine the circulating concentrations of CD31+/CD42, CD62E+ and CD34+ MPs in chronic stroke subjects, focusing on the effects of chronic stroke by comparing them to both older adults without a history of stroke but with T2DM, as well as older and young healthy controls. Plasma from three groups of sedentary older (50–75 years) men and women (chronic stroke, T2DM or older healthy) as well as a group of younger (18–39 years) healthy controls was isolated from fasting blood and CD31+/CD42b-, CD62E+ and CD34+ MPs were quantified using flow cytometry (n=17/group). Concentrations of CD31+/CD42b- and CD62E+ MPs were higher in the T2DM group (P<0.05), but not chronic stroke, compared to older and younger healthy adults. CD62E+ MP and CD34+ MPs concentrations were elevated in the older compared to younger adults (P<0.05 for both). Sub-analyses excluding chronic stroke subjects who were also diagnosed with diabetes [stroke(diabetes-)] revealed lower CD31+/CD42b- (P<0.05) and CD62E+ (P=0.08) MPs in the stroke(diabetes-) group compared to the T2DM group. CD31+/CD42b- MPs and CD62E+ MPs concentrations were each associated with fasting glucose levels and CD31+/CD42b- MPs also were associated with triglyceride levels. As MPs have been proposed as potential targets for diagnosing, treating and identifying the clinical progression of T2DM, our study provides further support for the use of CD31+/CD42b- and CD62E+ MPs in the clinical progression of T2DM and associated vascular complications.

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AUTHOR CONTRIBUTIONS

RQL and SJP conceived and designed the research project; RQL, MCS, JBB, ASR, CEH and SJP collected data and performed experiments; RQL and SJP analyzed data, RQL and SJP interpreted results, RQL prepared figures and drafted the manuscript; RQL, MCS, JBB, ASR, CEH and SJP edited, revised and approved the final version of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

Keywords

endothelial microparticle; diabetes mellitus; stroke; older age

INTRODUCTION

Cardiovascular disease (CVD) including stroke is the leading cause of death in developed countries but only ~50% of CV events can be explained by traditional biomarkers such as hypercholesterolemia and hypertension (Heidenreich *et al.*, 2011). Plasma microparticles (MPs), small (~0.1 – 1µm) vesicle-like structures formed from the plasma membrane of endothelial cells and other blood-related cells in response to apoptosis or endothelial cell activation (Dignat-George & Boulanger, 2011; Paudel *et al.*, 2016), were recently identified as potential novel biomarkers for CVD risk. MPs express surface markers of their parent cell and are physiologically relevant in chronic stroke because they contain factors such as microRNAs, mRNA, and proteins that can be transferred to other cell types to promote both deleterious or beneficial effects such as endothelial activation and inflammation or endothelial differentiation and survival, respectively (Puddu *et al.*, 2010; Dignat-George & Boulanger, 2011; Paudel *et al.*, 2016). Elevations in MP levels may provide important clinical information regarding the extent of vascular disruption in individuals with chronic stroke. For example, endothelial vasoreactivity as measured by flow-mediated dilation is inversely related to plasma MP levels, independent of age (Esposito *et al.*, 2006b; Chironi *et al.*, 2009). Furthermore, greater carotid-femoral pulse wave velocity measures of arterial stiffness are associated with higher circulating MP concentrations in healthy adults (Wang *et al.*, 2007). Thus, MPs serve as both biomarkers of vascular stress and potentially promote downstream signaling through transport of biological material (Sabatier *et al.*, 2002; Lacroix *et al.*, 2007; Abid Hussein *et al.*, 2008; Chironi *et al.*, 2009; Dignat-George & Boulanger, 2011) or interaction with membrane bound cellular receptors (Zhang *et al.*, 2015).

Although the precise mechanisms contributing to MP release are not completely understood, the circulating concentration and type of MP (i.e. surface markers present) may provide an index of the extent and nature of cellular damage, respectively. For example, one subset of MPs, CD31+/CD42b- MPs, contain endothelial surface markers of non-platelet origin, which *in vitro* studies indicate are shed by endothelial cells as a result of apoptosis (Horstman *et al.*, 2004; Chironi *et al.*, 2009). High circulating concentrations of CD31+/CD42b- MPs are associated with clinical conditions including hypertension (Preston *et al.*, 2003; Viera *et al.*, 2012) and play a major role in the atherosclerotic process (Paudel *et al.*, 2016). CD62E+ MPs, on the other hand, although also of endothelial origin, are released in response to cellular activation (for example, exposure to elevated levels of TNF-α). While CD62E+ MPs are present even in healthy individuals (Berkmans *et al.*, 2001), elevated MP numbers are seen in various cardiometabolic conditions and in association with cardiovascular risk (Lee *et al.*, 2012; Babbitt *et al.*, 2013). Indeed, Lee et al. found that high CD62E+ MP number in chronic stroke survivors was associated with greater risk of major cardiovascular events and hospitalization within 3 years after their stroke (Lee *et al.*, 2012). Finally, CD34+ MPs are derived from hematopoietic progenitor and endothelial cells. The CD34 surface marker on hematopoietic cells is often associated with pro-angiogenic

functions. Like CD34+ hematopoietic cells, CD34+ MPs are elevated in acute coronary syndrome patients compared to those adults with stable angina and healthy controls (Stepien *et al.*, 2012). Despite the few studies suggesting CD34+ MPs as biomarkers with potential pro-angiogenic properties, this MP population has not been well explored. Given the potential roles of these 3 types of circulating MPs in maintaining vascular homeostasis, the study of these MPs in people with chronic stroke and associated risk factors is warranted.

Stroke is particularly prevalent in older adults and is associated with a number of other cardiometabolic complications. Along with macrovascular complications associated with chronic stroke (Ivey *et al.*, 2010), skeletal muscle atrophy of the paretic limb and associated reductions in skeletal muscle capillarization (Prior *et al.*, 2009) often lead to the development of insulin resistance (Prior *et al.*, 2009) and reduced cardiovascular fitness (Ryan *et al.*, 2000; Billinger *et al.*, 2012). Indeed, more than 75% of individuals with chronic stroke have impaired glucose tolerance or type 2 diabetes (T2DM) (Ivey *et al.*, 2006). While there are several studies that have examined the effects of an acute stroke on different MP populations (Cherian *et al.*, 2003; Simak *et al.*, 2004; Simak *et al.*, 2006; Williams *et al.*, 2007; Jung *et al.*, 2009; Chiva-Blanch *et al.*, 2016), only one, to our knowledge, has looked at the MP response in chronic stroke survivors. Lee *et al.*, found that high circulating CD62E+ MPs are associated with greater CVD risk in chronic stroke (Lee *et al.*, 2012) but it is unknown whether this occurs independently of older age or insulin resistance. The purpose of this study was to determine circulating concentrations of CD31+/CD42b-, CD62E+ and CD34+ MPs in chronic stroke patients compared with healthy, young controls, while accounting for potential effects of age and T2DM by also studying groups of healthy older adults and older adults with T2DM. We hypothesized a graduated effect with chronic stroke patients having the highest MP concentrations, followed by T2DM, healthy older and healthy younger controls. We then sought to determine whether traditional CVD risk factors (i.e., plasma lipoprotein-lipid and glucose concentrations) were associated with concentrations of each MP subtype. A greater understanding of factors associated with older age with and without cardiometabolic disease may help establish MPs as novel clinical biomarkers for CVD risk.

METHODS

Ethical Approval

The University of Maryland College Park (UMCP) and the University of Maryland School of Medicine (UM-SOM) Institutional Review Boards approved all study procedures (UM-SOM: #HP00041347, HP00047188, HP00057124, and UMCP: # 343286) and all subjects provided informed verbal and written consent. Study procedures were carried out in accordance with the Declaration of Helsinki except for registration in a research database.

Screening and standard assessments

Subjects were recruited from the greater Baltimore and Washington DC area. All subjects were sedentary completing 20 min of exercise on 2 days per week. Chronic stroke, T2DM and older healthy subjects were sedentary men and women 50–75 years old (the latter postmenopausal for >2 years and not prescribed hormone replacement therapy).

Chronic stroke subjects had suffered an ischemic stroke >3 months prior to testing and had completed all conventional physical therapy. T2DM subjects either had a diagnosis of type 2 diabetes, a HbA_{1C} value of >6.5%, or a fasting glucose >126 mg/dL on two or more occasions (Association, 2018). Chronic stroke and T2DM subjects were not excluded due to other chronic conditions including high blood pressure, hyperlipidemia or hyperglycemia and these conditions and accompanying medications were documented. Healthy older adults were of similar age to the chronic stroke and T2DM groups but had blood pressure, and plasma lipid and glucose levels in the normal ranges. Younger adults were sedentary men and women 18–39 years old. Younger women were all tested during the early follicular phase of their menstrual cycle. Exclusion criteria for the healthy groups were: smoking, known CVD, type 1 or type 2 diabetes, chronic obstructive pulmonary disease, current treatment for active cancer, systolic blood pressure \geq 130 mmHg; diastolic blood pressure \geq 90 mmHg; serum total cholesterol \geq 200 mg/dL; low density lipoprotein-cholesterol (LDL-C) \geq 130 mg/dL; high-density lipoprotein-cholesterol (HDL-C) \leq 35 mg/dL; or fasting glucose \geq 100 mg/dL.

Peak Oxygen Consumption

Peak oxygen consumption (VO_{2peak}) was measured by indirect calorimetry (Quark, Cosmed USA, Chicago, IL) during a graded treadmill exercise test on a motorized treadmill. Subjects walked at a constant velocity with the grade initially set to 0% and increasing 4% after the first 2 minutes and 2% every 2 min thereafter to volitional exhaustion, as reported previously (Ivey *et al.*, 2007; Prior *et al.*, 2009). Tests were terminated at the subject's request or according to criteria set forth by American College of Sports Medicine (Pescatello *et al.*, 2014). Subjects were allowed handrail support but were instructed to minimize handrail use to that necessary to maintain balance. VO_{2peak} was defined as the highest oxygen consumption value obtained in the last minute of exercise.

Fasted blood draw

Subjects reported to the lab the morning after an overnight (12-hr) fast. Subjects were asked to refrain from food, alcohol, caffeine until after the blood draw. Height, weight and seated blood pressure were recorded after arriving to the lab. Blood was collected in tubes containing 15% potassium EDTA; plasma was isolated for measurement of lipoprotein-lipid levels and plasma for MP measures was isolated and stored at -80° C until future analysis.

Blood sample analyses

Plasma glucose levels were measured in duplicate using the glucose oxidase method (2300 STAT Plus, YSI, Yellow Springs, Ohio, USA). Plasma triglyceride (TG) and cholesterol were analyzed by enzymatic methods (Hitachi mofel-917 analyzer). HDL-C was measured in the supernatant after precipitation with dextran sulfate and LDL-C was calculated using the Friedewald equation (Friedewald *et al.*, 1972): $[LDL-C] = [total\ cholesterol] - [(TG/5 + HDL-C)]$

Plasma microparticles

MP quantification and analysis was performed as previously described (Jenkins *et al.*, 2011) with minor modifications. Briefly, plasma samples were thawed and platelet-poor plasma was obtained through centrifugation. Platelet-poor plasma was then further centrifuged to obtain cell-free plasma (CFP), which was used for MP analyses. 50uL of CFP was incubated with anti-CD31-phycoerythrin (PE) and anti-CD42b-fluorescein isothiocyanate (FITC), anti-CD62E-PE or anti-CD34-FITC (BD Biosciences) for 30 minutes. Samples were then fixed with 2% paraformaldehyde and diluted with filtered PBS before flow cytometric analysis.

Microparticle Quantification and Analysis

MP quantification was performed using an LSR II flow cytometer with a lower detection limit of 0.5 μm . Samples were each analyzed for 3 minutes at a medium flow rate. Countbright Absolute Counting Beads (ThermoFisher Scientific) were added to each sample immediately before quantification and ultra-pure water was run for 1 minute in between each sample. MPs were defined as CD31+CD42b-, CD62E+ or CD34+ events between 0.5–1.0 μm , excluding exosomes and other small microvesicles from analyses (Gould & Raposo, 2013). A logarithmic scale was used for forward scatter signal, side scatter signal and each fluorescent channel and 0.9 μm standard precision NIST traceable polystyrene particle beads (Polysciences, Inc) were used for size calibration and forward scatter signal. Proper color and size controls were used to distinguish true events from background noise. Examples of controls and gating strategies can be found in Figure 1. MPs were quantified using Winlist 6.0 (Verity Software). The concentration of MPs was calculated using Countbright Absolute Counting Beads (ThermoFisher Scientific).

$$\frac{\text{Number of EMP events}}{\text{Number of Countbright events}} = \frac{\text{Countbright Concentration}}{\text{Volume of sample}}$$

Statistical Analysis

Statistical analyses were completed using IBM SPSS version 21 (Armonk, NY). Assumptions of homoscedasticity and normality were evaluated for all measures. MP data were log-transformed for statistical analyses due to a non-normal distribution of residuals in raw data. Differences among groups were compared using ANOVAs. For our main analyses, power calculations using previous cross-sectional comparisons of MPs in populations with various levels of CVD risk factors (Preston *et al.*, 2003; Lansford *et al.*, 2016) estimated a sample size of n=11/group to yield detectable differences and 80% power. As forty-one percent of chronic stroke subjects were also diagnosed with diabetes mellitus, a sub-analysis was performed to distinguish effects of chronic stroke and T2DM by dividing the chronic stroke group into stroke(diabetes+) and stroke(diabetes-). Pearson correlation coefficients were calculated for log values of plasma MP and values of physical fitness, blood lipids, blood pressure, body mass index (BMI), and glucose. Factors that significantly correlated with MPs were further assessed in a multivariable regression. Statistical significance was accepted at P 0.05. MP data are expressed as geometric means and 95% confidence limits; remaining data are expressed as means \pm standard deviation.

RESULTS

Subject characteristics

Subject characteristics can be found in Table 1. By design, the chronic stroke, T2DM and healthy older groups were significantly older than the younger group; these three groups also had higher BMI values compared with the younger adults ($P < 0.05$). There were no significant differences in age or BMI among the three older groups (chronic stroke, T2DM and healthy older). All older groups exhibited significantly lower absolute and relative $VO_{2\text{peak}}$ compared with younger adults, with chronic stroke subjects having the lowest $VO_{2\text{peak}}$ values ($P < 0.05$). Both HbA1c and fasting glucose were highest in the T2DM group and all three older groups exhibited significantly higher glucose levels compared with healthy younger adults. HbA1c levels were $7.5 \pm 0.85\%$ in the stroke(diabetes+) group and $5.5 \pm 0.12\%$ in stroke(diabetes-) group, which are similar to their T2DM and healthy older counterparts, respectively. The T2DM group had significantly higher triglycerides and significantly lower HDL-C levels compared with both healthy younger and older adults ($P < 0.05$ for both). All subjects in the chronic stroke group and T2DM group were on at least one statin and/or blood pressure medication and 88% of subjects diagnosed with T2DM were prescribed medications to control diabetes (Table 2).

Microparticle Concentrations

CD31+/CD42b- Microparticles—CD31+/CD42b- MP concentration was relatively low in young adults and not different in healthy older adults ($P = 0.13$); however, CD31+/CD42b- MP concentrations from chronic stroke subjects were 170% higher than younger adults ($P = 0.04$). Additionally, T2DM subjects had 384% and 204% greater CD31+/CD42b- MP concentrations than healthy young ($P = 0.008$) and older adults ($P = 0.02$), respectively (Figure 2A). Analyses of chronic stroke subjects with and without diabetes indicated that the stroke(diabetes+) had 105% higher CD31+/CD42b- MP concentrations compared with stroke(diabetes-), although this did not reach statistical significance ($P = 0.20$). However, sub-analyses revealed that the T2DM group had 140% more CD31+/CD42b- MPs compared with chronic stroke(diabetes-) group ($P = 0.03$; Figure 3A).

CD62E+ Microparticles—Compared with young adults, CD62E+ MP concentrations were significantly higher in all older groups (122%, 179% and 393%, in healthy ($P = 0.03$), chronic stroke ($P = 0.02$) and T2DM subjects ($P = 0.005$), respectively; Figure 2B). Furthermore, CD62E+ MP concentrations were higher in the T2DM group compared with healthy older adults ($P = 0.03$, Figure 2B). Analyses of chronic stroke subjects with and without diabetes indicated that the stroke(diabetes+) had 37% more CD62E+ MPs compared with stroke(diabetes-), although this did not reach statistical significance ($P = 0.23$). Sub-analyses indicated a tendency for 101% higher CD62E+ MPs in the T2DM compared with the stroke(diabetes-) ($P = 0.08$; Figure 3B).

CD34+ Microparticles—There was an effect of age on CD34+ MP concentrations, with healthy older adults ($P = 0.02$), chronic stroke ($P = 0.01$), and T2DM ($P = 0.01$) subjects having significantly higher CD34+ MP concentrations than healthy younger individuals (61%, 47% and 99% higher, respectively; Figure 2C). No additional effect of T2DM or chronic stroke

was observed ($P=0.36$ and $P=0.38$ for both). CD34+ MP concentrations did not differ between stroke subjects with and without diabetes and sub-analyses of stroke(diabetes-) were consistent with findings from original analyses ($P=0.92$; Figure 3C).

Relationships among MP concentrations and CVD risk factors

CD31+/CD42b- MP concentrations directly correlated with glucose, triglycerides and BMI ($r = 0.35-0.55$; $P<0.001$ for all) and inversely correlated with HDL cholesterol ($r = -0.35$; $P<0.001$). When these factors were entered into a regression analysis, significant correlations between only CD31+/CD42b- MPs and glucose and triglycerides persisted (Figure 4A–B; $P<0.001$ for both). Similarly, CD62E+ MP concentrations significantly correlated with plasma glucose, triglycerides and BMI ($r = 0.32-0.46$; $P<0.001$ for all) and inversely with HDL cholesterol ($r = -0.32$; $P<0.001$). When these factors were entered into a regression analysis only glucose remained significantly correlated with CD62E+ MP concentrations (Figure 4C; $P<0.001$). There was no significant correlation between and CD34+ MP concentration and any factor measured ($r = | 0.03-0.18 |$; $P>0.05$ for all).

DISCUSSION

MPs are potential biomarkers for endothelial impairment in older age and chronic disease; however, it was previously unknown whether the chronic effects of stroke, T2DM or older age account for elevations in MPs. In this study, we demonstrate that elevated concentrations of CD31+/CD42b- and CD62E+ MPs appear to be driven by T2DM but not chronic stroke. We also show that older age results in further elevations in CD62E+ MP concentrations, while CD34+ MP concentrations are affected by older age alone. Additionally, we found that CD31+/CD42b- MPs and CD62E+ MPs concentrations were each associated with fasting glucose levels and that CD31+/CD42b- MPs also were associated with triglyceride levels. This is not surprising, as high plasma glucose and triglyceride levels coincide with level of T2DM control and are associated with increased CVD risk. Our findings provide further support for the potential use of MPs as novel biomarkers for assessing the clinical progression of CVD risk in older age.

Chronic stroke is associated with long-term vascular consequences that extend far beyond the cerebrum into the periphery (Alexandrova & Bochev, 2005; Billinger *et al.*, 2012; Banerjee & Chimowitz, 2017) and include reduced endothelial vasoreactivity (Ivey *et al.*, 2010) and capillary rarefaction (Prior *et al.*, 2009), most notably in the paretic limb. These vascular complications could, in part, be attributed to MPs with pro-inflammatory effects as we hypothesized that chronic stroke would have elevated MP concentrations compared with other groups. As expected, all three MP subtypes were higher in the chronic stroke group compared with the younger adults. However, we found no differences in any of the three MP subtypes between chronic stroke and the other two older groups (T2DM or healthy older) in our primary analyses. In this study, the degree of hemiparesis was not quantified in the individuals with chronic stroke, but it is possible that MP and other outcomes could vary within this population as a function of the degree of hemiparesis. Impaired glucose metabolism is highly prevalent in chronic stroke (Ivey *et al.*, 2007; Prior *et al.*, 2009) and 42% of the subjects in our chronic stroke group were also diagnosed with type 2 diabetes.

Therefore, we performed additional analyses in which we excluded all chronic stroke subjects who were also diagnosed with diabetes (i.e., stroke(diabetes-) vs. T2DM group). Interestingly, the stroke(diabetes-) group had significantly lower CD31+/CD42b- and there was a trend for lower CD62E+ MPs compared with the T2DM group. Our findings indicate that the presence of T2DM, and not the effects of chronic stroke itself, appears to explain the elevations in CD31+/CD42b- and CD62E+ MPs in the present report.

T2DM is associated with disturbances in metabolism and vascular function (Deng *et al.*, 2016) which could be partially attributable to concentrations of specific MPs. We found that CD31+/CD42b- and CD62E+ MPs were highest in subjects with T2DM compared with healthy older and younger groups. Other studies have also found that CD31+/CD42b- MP levels are higher in men with T2DM compared with controls (Esposito *et al.*, 2007; Leroyer *et al.*, 2008) and that they are predictive of macrovascular complications in T2DM (Jung *et al.*, 2011). However, there are currently few studies demonstrating alterations in CD62E+ MPs in subjects with T2DM (Esposito *et al.*, 2007). Our findings suggest that MPs associated with endothelial apoptosis and activation are more elevated in T2DM than in chronic stroke or older age alone. MPs are released in response to a variety of inflammatory stimuli including hyperglycemia and dyslipidemia (Dignat-George & Boulanger, 2011; Jansen *et al.*, 2013) and a chronic inflammatory environment in the setting of T2DM may promote the development and release of these MPs. Once released, MPs may further exacerbate this condition by transporting bioactive cellular content to other cell types or binding to other target cell receptors and interfering with cell signaling. Indeed, one study found that MPs promote insulin resistance by influencing insulin signaling pathways or by interfering with glucose transporter-4 translocation and blocking glucose uptake by the cell (Zhang *et al.*, 2015). Thus, elevations in CD31+/CD42b- and CD62E+ MPs may be a result of, as well as a contributor to, T2DM and its complications.

While various MP concentrations have been implicated as a consequence and potential contributor to various age-related cardiometabolic diseases, the concentrations of different MPs in older vs. younger adults has not been well explored. Animal studies in mice demonstrate that older mice exhibit more leukocyte-derive MPs compared with young mice (McDonald *et al.*, 2010), but studies in non-human primates found that age does not correlate with the number of different endothelial MPs (Knight *et al.*, 2013). Similarly, Gustafson *et al.*, found no relationship between age and CD62E+ MP concentrations (Gustafson *et al.*, 2015) in healthy adults. To our knowledge, this is the first study to demonstrate elevations in CD62E+ and CD34+ MP concentrations in older compared with younger adults. These findings suggest that endothelial activation resulting in the release of CD62E+ MPs may contribute to the age-related decline in vascular function. In the present report, all healthy older adults were sedentary. As regular aerobic exercise training can attenuate the age-related decline in endothelial function (Seals *et al.*, 2011; DeVan & Seals, 2012), future studies should investigate whether elevations in CD62E+ and CD34+ MPs are mitigated in older chronically active adults compared with sedentary adults.

The origin and effect of CD34+ MPs has been less explored in the literature compared with other MP populations. CD34+ is best known as a progenitor cell marker but can also be present on endothelial cells. In an atherogenic environment, CD34+ progenitor cells can take

on characteristics of foam cells in the development of atherosclerosis, which may result in MP release (Daub *et al.*, 2006). Thus, elevations in CD34+ MPs observed with in the older groups may be indicative of chronic low-grade inflammation associated with older age. Alternatively, CD34+ exosomes have been found *in vitro* to contain angiogenesis-promoting miRNAs (Sahoo & Losordo, 2014; Mathiyalagan *et al.*, 2017). Although the size and mechanisms of release differ between exosomes and MPs, potential similarities in cargo between the two extracellular vesicles suggest that perhaps the elevations in CD34+ MPs are serving in a compensatory capacity to offset the elevations in CD62E+ MPs also associated with older age. Our findings suggest a need for further studies to better clarify the role of CD34+ MPs observed with older age.

In an effort to gain insight into systemic factors that may be involved in MP release from the endothelium, we performed regression analyses among the three MP subtypes and levels of physical fitness, plasma lipids, BMI, and glucose, and found that CD31+/CD42b- and CD62E+ MPs directly correlated with fasting glucose levels. These findings are congruent with previous *in vitro* work demonstrating that a high glucose environment induces endothelial cell apoptosis (Baumgartner-Parzer *et al.*, 1995) and endothelial activation (Videm & Albrigtsen, 2007) as well as *in vivo* findings that hyperglycemia is associated with greater CVD risk (Tabit *et al.*, 2010). Additionally, we found that triglyceride levels directly correlated with CD31+/CD42b- MP concentrations, which supports hypertriglyceridemia's known association with endothelial impairments (Lundman *et al.*, 2003). The present findings are congruent with other reports of direct associations between CD31+/CD42b- MP and triglyceride levels (Ferreira *et al.*, 2004; Amabile *et al.*, 2014), and extend those findings by demonstrating elevated MPs in subjects with more chronic, lower-grade elevations in fasting triglycerides. Conversely, regression analyses found no association between CD62E+ and fasting triglyceride levels. In the current study, the T2DM group exhibits the highest triglyceride and glucose levels of all groups. Although triglyceride levels are still within the normal range, this is likely pharmacologically induced as all T2DM subjects were prescribed statins, potentially influencing our results. It seems likely that a combination of various metabolic imbalances (elevated glucose, triglycerides and lower HDL-C) contribute to endothelial apoptosis which lead to greater MP release in the T2DM group.

There are several differences between the current and previous studies on MPs. First, subjects in our study represent a wider range of age and health including chronic stroke and T2DM. Additionally, it is important to note that 76% of chronic stroke subjects and all T2DM subjects in our study were on some form of diabetes medication, statin or lipid lowering medication. Some medications have been found to influence specific MP concentrations with studies indicating that statins both increase (Diamant *et al.*, 2017) or decrease (Tramontano *et al.*, 2004) different MPs *in vitro* and some medications resulting in lower MP concentrations in individuals with diabetes and hypertension (Nomura *et al.*, 2005; Esposito *et al.*, 2006a; Nomura *et al.*, 2007; Nomura *et al.*, 2009). Due to their necessity in these populations we were unable to withhold medications for longer than the 12-hour fasting period and the use of medications may have influenced some of our findings. More research is needed to better understand the effects of individual medications and the combined effects of multiple medications on MP concentrations. Finally, it is worth noting

that HDL-C levels in our subjects were surprisingly high, especially in the chronic stroke group, with only T2DM subjects having significantly lower HDL-C levels compared with healthy younger adults. This is likely a result of statin usage in the chronic stroke population. HDL cholesterol is involved in the transport of cholesterol from the arteries; thus, it is possible that the relatively high HDL-C levels attenuated endothelial impairment and MP production in the chronic stroke population.

A strength of this study is the balance for sex across groups, although the relatively low female participation prevented our ability to examine sex differences in MPs concentrations from each group. Some recent studies have begun to examine these issues, although the sex differences remain unclear with some studies reporting lower and others higher resting levels of CD62E+ MPs in sedentary younger women compared with men (Durrer *et al.*, 2015; Gustafson *et al.*, 2015). In addition, some studies are beginning to explore MP responses to an acute bout of aerobic exercise and find differential responses between men and women in CD62E+ (Durrer *et al.*, 2015; Lansford *et al.*, 2016; Shill *et al.*, 2018) and CD34+ MPs (Lansford *et al.*, 2016), suggesting sex differences in responses to vascular stressors with exercise. Thus, future studies specifically designed to examine mechanisms related to sex differences in various populations and in response to an exercise or nutritional intervention might provide better indication as to the use of MPs as sex-specific biomarkers of CVD risk.

Because the present study was limited to correlation analyses of relationships among CD31+/CD42b- and CD62E+ MP concentrations and triglycerides or glucose, it cannot provide specific mechanistic evidence of the effects of these factors on MPs (or vice versa). Future studies are needed to investigate molecular pathways involved in dyslipidemia and hyperglycemia, how these relate to MP production, and potential mechanisms through which MPs may further be involved in disease pathology to fully understand the complex roles of different MP subtypes. Additionally, we are unable to present the timeframe since diagnosis of T2DM for all our subjects, which might play a role in the extent of vascular stress and consequently, MP concentration. Future work documenting the concentration of different MP subtypes at different stages of T2DM diagnosis would allow for a better understanding of the disease process and how MPs are involved. Recent work has demonstrated that diet, specifically low-carbohydrate diet, can affect MP concentrations in T2DM (Francois *et al.*, 2018). Subjects in the current study arrived fasted but diet was not controlled leading up to their visit which may have affected our outcomes. This current study was designed as a cross-sectional analysis of the levels of three MP subtypes. Further classification of MPs including analyses of MP content and activity as well as their direct effects on endothelial function and whether this can be modified are necessary to fully understand the nature and actions of these factors. It should be acknowledged that plasma was obtained using EDTA anticoagulant. Although ACD anticoagulant is preferred (Golanski *et al.*, 1996; Jayachandran *et al.*, 2012; Gyorgy *et al.*, 2014), we (Jenkins *et al.*, 2011) and others (Babbitt *et al.*, 2013) have successfully quantified MPs using EDTA plasma. Thus, while this may limit our ability to compare specific concentrations of MPs to some other studies, observations of trends for differences in MP levels among similar populations are possible. The samples used in this study were collected on different dates and stored frozen prior to analysis which can influence MP concentrations (Dey-Hazra *et al.*, 2010). The storage times varied within all groups, but were not likely different among groups. However, analyses of

MP degradation would allow for a better understanding of whether potential differences in storage times affected our outcomes. Finally, we acknowledge that the sub-analyses of stroke(diabetes-) included a lower sample size, and thus, may not be adequately powered. This may explain why significant differences between the stroke (diabetes+) and stroke (diabetes-) groups were not detected. While these findings may prove physiologically significant, larger studies are needed to confirm the effects of stroke with and without the presence of diabetes.

In conclusion, our study demonstrates that elevations in concentrations of CD31+/CD42b- and CD62E+ MP are much more pronounced in T2DM, but not chronic stroke and demonstrates, for the first time, elevations in CD62E+ and CD34+ MP concentrations in older vs. younger adults. Furthermore, we demonstrate relationships between fasting glucose and CD31+/CD42b- and CD62E+ MPs and triglycerides and CD31+/CD42b- MPs. As various MPs have been proposed as potential targets for diagnosing, treating and identifying the clinical progression and complications of T2DM, our study provides further support for the use of CD31+/CD42b- and CD62E+ MPs in examining these issues. Specific MPs, perhaps combined with other established biomarkers of vascular health such as endothelial progenitor cells, may eventually be used to determine the balance between endogenous vascular injury and repair in different populations to provide a better indicator of the clinical progression of CVD.

ACKNOWLEDGEMENTS

RQL was supported by the NIH T32 (HL007698). This study was supported by a Veterans Affairs (VA) Maryland Exercise and Robotics Center for Excellence (MERCE) Pilot Award (RQL), VA Senior Research Career Scientist Award (ASR), NIH R01-AG030075 (ASR), Career Development Award Number IK2 RX-000944 from the United States (U.S) Department of VA Rehabilitation R&D (Rehab RD) Service (MCS), K23- AG040775 (NIH and the American Federation for Aging Research; SJP), (Department of VA; SJP), and the Baltimore VA Geriatric Research, Education and Clinical Center (GRECC).

Abbreviations

BMI	body mass index
CVD	cardiovascular disease
FITC	fluorescein isothiocyanate
HDL-C	high density lipoprotein cholesterol
LDL-C	low density lipoprotein cholesterol
MP	microparticles
PE	phycoerythrin
T2DM	Type 2 Diabetes Mellitus
TG	triglyceride
VO₂peak	Peak oxygen consumption

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NEW FINDINGS**What is the central question of this study?**

What is the effect of chronic stroke on circulating microparticle populations, accounting for potential effects of age and type 2 diabetes?

What is the main finding and its importance?

Elevated concentrations of CD31+/CD42b- and CD62E+ microparticles appear to be driven by type 2 diabetes but not chronic stroke and are associated with fasting glucose and triglyceride levels. Older age results in elevations in CD62E+ and CD34+ microparticle concentrations. These microparticles have been proposed as potential targets for diagnosing, treating and identifying the clinical progression and complications of type 2 diabetes.

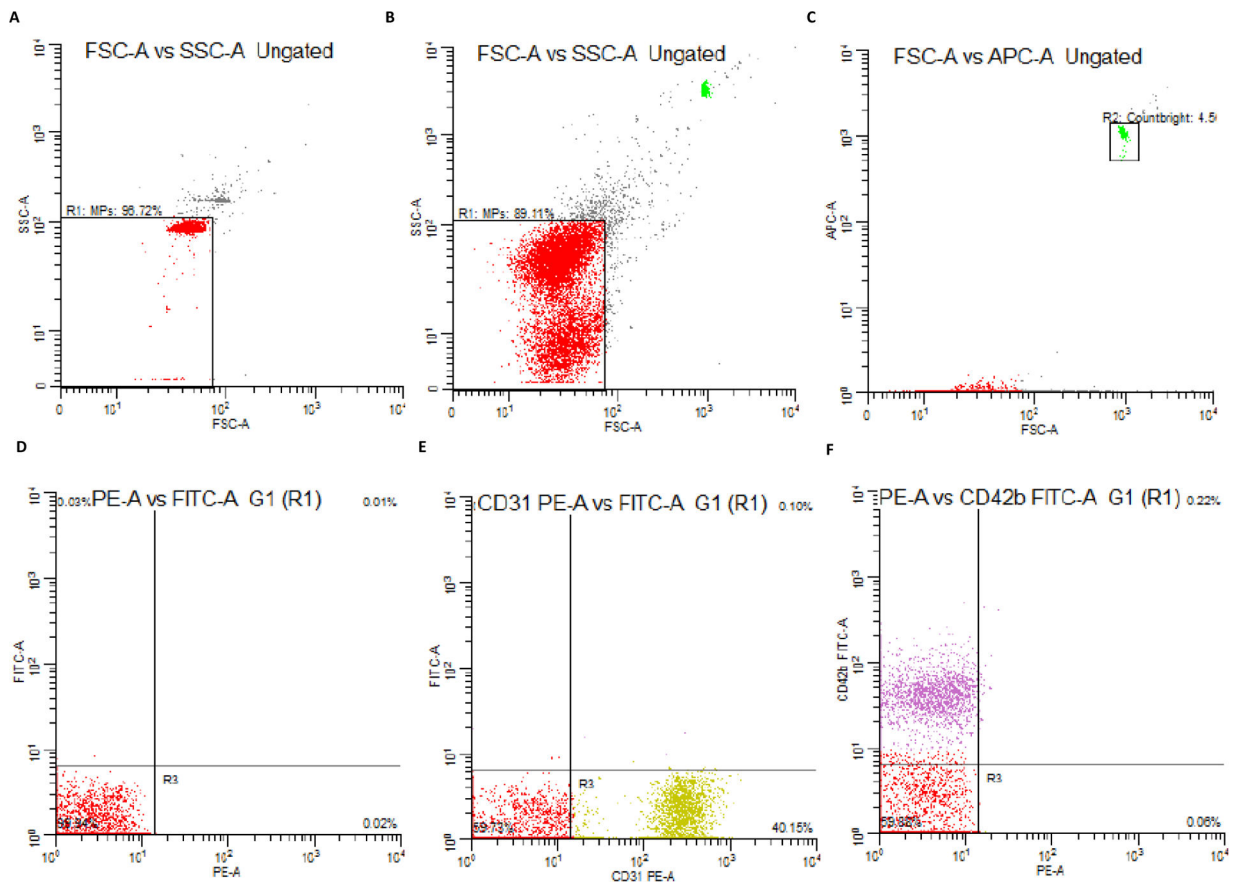


Figure 1.

Examples of gating strategies for flow cytometry analyses. a-b) NIST beads are used to create gates which delineate events $<1.0 \mu\text{m}$ c) Countbright beads are used to ensure a consistent number of events for each sample and are used to calculate MP concentration. d) unstained controls, e) PE color control and f) FITC color controls

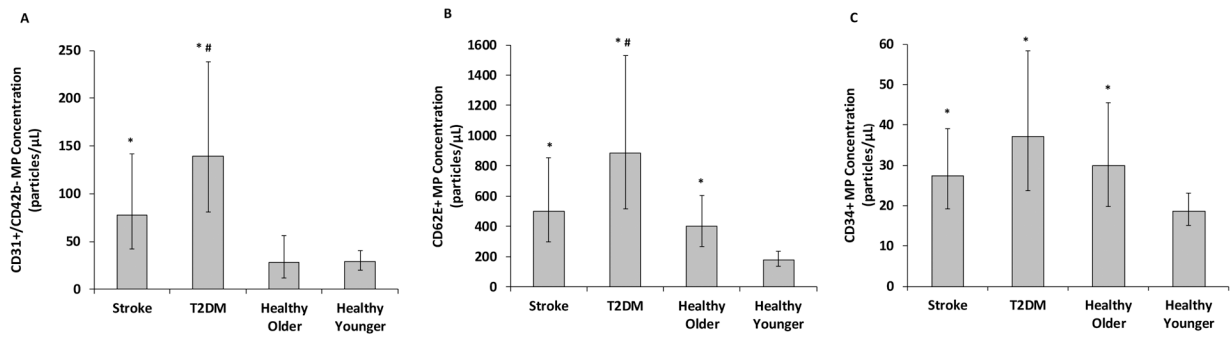


Figure 2.

Comparison of a) CD31+/CD42b-, b) CD62E+ and c) CD34+ microparticle (MP) concentrations in individuals with chronic stroke, type 2 diabetes mellitus (T2DM), and healthy older and younger individuals (n=17/group). Raw data are presented but were log-transformed for analyses. Figures represent mean and 95% confidence limits. * indicates significantly different than healthy younger adults (P<0.05); # indicates significantly different than healthy older adults (P<0.05)

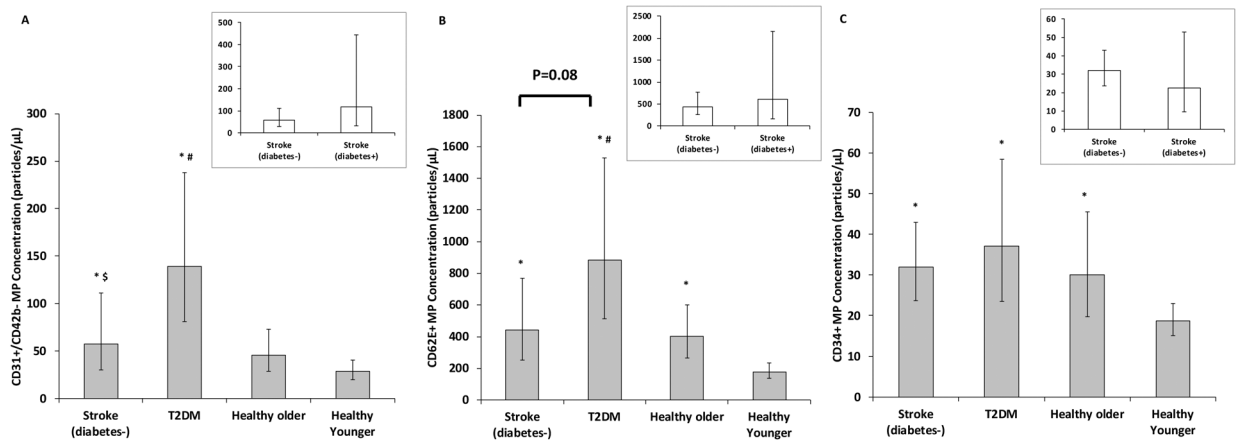


Figure 3.

Comparison of a) CD31+/CD42b-, b) CD62E+ and c) CD34+ microparticle (MP) concentrations in chronic stroke(diabetes-) (n=10), type 2 diabetes mellitus (T2DM)(n=17), and healthy older (n=17) and younger individuals (n=17). Inset figures represent comparison between chronic stroke(diabetes+) (n=7) and chronic stroke(diabetes-) (n=10). Raw data are presented but were log-transformed for analyses. Figures represent mean and 95% confidence limits. * indicates significantly different than healthy younger adults ($P < 0.05$); # indicates significantly different than healthy older adults ($P < 0.05$); \$ indicates significantly different than T2DM ($P < 0.05$)

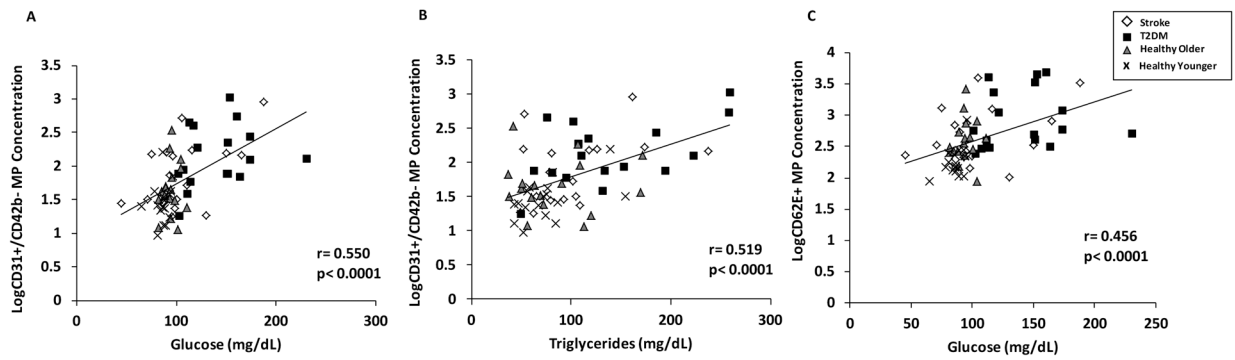


Figure 4.

Correlations between CD31+/CD42b- microparticles and a) fasting glucose or b) triglycerides, and c) between CD62E+ microparticles and glucose. Log-transformed MP data are presented.

Table 1.

Subject Characteristics

	Stroke (n=17)	T2DM (n=17)	Healthy Older (n=17)	Healthy Younger (n=17)
Sex (M/F)	13/4	14/3	13/4	14/3
Age (years)	65 ± 8.9 [*]	61 ± 6.2 [*]	62 ± 6.9 [*]	27 ± 4.6
Weight (kg)	90.1 ± 19.5	99.7 ± 22.9	92.8 ± 14.6	72.8 ± 11.2
BMI (kg/m ²)	29 ± 4.1 [*]	32 ± 5.7 [*]	30 ± 5.3 [*]	24 ± 2.8
HbA1c (%)	6.3 ± 1.8	7.2 ± 1.1 [#]	5.5 ± 0.3	-
Glucose (mg/dL)	107 ± 35.3 ^{*,§}	140 ± 34.4 ^{*,#}	94 ± 7.9 [*]	86 ± 7.3
Total cholesterol (mg/dL)	162 ± 69.8	155 ± 31.7 [#]	186 ± 30.2	172 ± 25.5
HDL-C (mg/dL)	50 ± 20.5	38 ± 11.8 ^{*,#}	51 ± 13.2	60 ± 20
LDL-C (mg/dL)	91 ± 54.8	88 ± 27.3 [#]	118 ± 23.8 [*]	97 ± 21.7
Triglycerides (mg/dL)	102 ± 50	138 ± 65.3 ^{*,#}	83 ± 42.3	74 ± 30.7
SBP (mmHg)	125 ± 16.1	131 ± 11 [#]	119 ± 17	124 ± 13.2
DBP (mmHg)	72 ± 8.2	76 ± 10.5	74 ± 7.8	78 ± 6.4
MAP (mmHg)	90.2 ± 8.2	94.6 ± 8.8	89.3 ± 8.8	93.1 ± 7.8
VO _{2peak} (L/min)	1.7 ± 0.7 ^{*,§#}	2.5 ± 0.6 [*]	2.5 ± 0.6 [*]	3.2 ± 0.9
VO _{2peak} (ml/kg/min)	18.7 ± 4.5 ^{*,§#}	25 ± 4.9 [*]	26 ± 7.5 [*]	44 ± 8.9

Means ± SD

^{*} significantly different than healthy younger (P<0.05)[#] significantly different than healthy older (P<0.05)[§] significantly different than T2DM (P<0.05)

Table 2.

Medications

	Stroke	T2DM	Healthy Older	Healthy Younger
Medication, n (%)				
Statin	9 (53)	11 (65)	1 (4)	0
Other lipid medication	4 (24)	6 (35)	0	0
Blood pressure medication	14 (82)	10 (59)	1 (4)	0
Diuretic	2 (12)	2 (12)	0	0
Diabetes medication	7 (41)	14 (82)	0	0
Insulin	2 (12)	3 (18)	0	0
Blood thinner	5 (29)	4 (24)	0	0
Heartburn/GERD medication	3 (18)	4 (24)	1 (4)	0
Aspirin	10 (59)	6 (35)	2 (12)	0
Other NSAID	4 (24)	4 (24)	3 (18)	0
Bronchodilator	2 (12)	0	2 (12)	0
Antidepressant	6 (35)	2 (12)	0	0
Anti-seizure medication	4 (24)	0	0	0
Glaucoma medication	2 (12)	3 (18)	0	0
Muscle relaxant	3 (18)	1 (4)	0	0
Bladder/kidney medication	2 (12)	2 (12)	0	0
Prostate hypertrophy medication	3 (18)	1 (4)	0	0
Prednisone	1 (4)	1 (4)	0	0

Data represent total number of subjects taking each medication and percent of total from each group; n (%)