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Original Article

Early endothelial damage detected by circulating particles in baboons fed a diet high in simple carbohydrates in conjunction with saturated or unsaturated fat

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Abstract: Studies have shown that high-fat diets cause blood vessel damage, however, assessing pathological effects accurately and efficiently is difficult. In this study, we measured particle levels of static endothelium (CD31+ and CD105+) and activated endothelium (CD62E+, CD54+ and CD106+) in plasma. We determined individual responses to two dietary regimens in two groups of baboons. One group (n = 10), was fed a diet high in simple carbohydrates and saturated fats (the HSF diet) and the other (n = 8) received a diet high in simple carbohydrates and unsaturated fats (the HUF diet). Plasma samples were collected at 0, 3, and 7 weeks. The percentages of CD31+ and CD62E+ particles were elevated at 3 weeks in animals fed either diet, but these elevations were statistically significant only in animals fed the HUF diet. Surprisingly, both percentages and counts of CD31+ particles were significantly lower at week 7 compared to week 0 and 3 in the HSF group. The median absolute counts of CD105+ particles were progressively elevated over time in the HSF group with a significant increase from week 0 to 7; the pattern was somewhat different for the HUF group with significant increase from week 3 to 7. The counts of CD54+ particles exhibited wide variation in both groups during the dietary challenge, while the median counts of CD106+ particles were significantly lower at week 3 than at week 0 and week 7. Endothelial particles exhibited time-dependent changes, suggesting they were behaving as quantifiable surrogates for the early detection of vascular damage caused by dietary factors.

Keywords: Circulating endothelial particles, vascular damage, dietary challenge, biomarkers, nonhuman primate model

Introduction

Diet composition is a key determinant of risk of cardiovascular disease [1], including the development and progression of multiple vascular complications [2]. Detrimental dietary components can stress or damage the endothelium lining the blood vessels. If circulating factors derived from detrimental dietary components persist for a period of time, they can lead to endothelial cellular blebbing and shedding of membrane fragments into the circulation. These particles vary in size, content, and mechanism of formation [3]. Elevated levels of endothelial particles have been observed when cells were exposed to environmental stimuli; for example, Combes and his colleagues found that cultured endothelial cells shed particles after TNF-α treatment [4]. Subsequent investigations established that the shedding of mic-

Table 1. Composition of chow and the two experimental diets

Diet	Carbohydrates	Proteins	Fats			
			Saturated	Unsaturated	Trans	Cholesterol
Chow	66	20	2.9	11.0		0.0059
HSF	45	15	17.4	22.0	1.0	0.0118
HUF	45	15	3.5	37.1	-	0.0118

All nutrients, except cholesterol, expressed as % of total calories; cholesterol expressed as % of dry weight.

roparticles from the plasma membrane was a common feature of activated vascular endothelium [5-7]. Accumulating evidence suggests that these particles can serve as biomarkers of endothelial dysfunction associated with pathogenesis of cardiovascular disease [8-10]. Although it is clear that circulating particles have been useful for investigating endothelial integrity consequent to dietary intervention [11], the diagnostic value of endothelial particles has remained unclear due to technological limitations and lack of standardized methods for particle detection and assessment.

Previously, we reported that a high-fat diet causes dysfunction and senescence of the arterial endothelium of baboons [12, 13]. Extrapolating from those results, we reasoned that the dysfunctional endothelium might have released endothelial cell fragments as particles into circulation and we hypothesized that circulating particles reflect pathological effects of high-fat diets on blood vessels. Here, we test our hypothesis in a prospective longitudinal study with serial measures of circulating particles rigorously evaluated over time using a non-human primate model, the baboon. In this investigation, we determined whether simple carbohydrates in conjunction with either saturated or unsaturated fats damaged the endothelium, and whether the two diets exerted different effects. Using this primate model, we sought to determine the biological effect of dietary simple carbohydrates in combination with high levels of saturated or unsaturated fats on levels of circulating endothelial-derived particles as potential surrogate indicators of vascular injury [9, 14]. We report here dynamic changes in circulating particles during a 7-week dietary challenge of two atherogenic diets. Our results demonstrate that endothelial particles exhibit time-dependent changes within weeks of challenge with atherogenic diets; suggesting that endothelial particles are quantifiable indicators of vascular damage by caused by dietary factors.

Materials and methods

Animals

Adult male baboons (*Papio* Sp.), aged from 6.1 to 7.4 years old, were used for this experiment, which was conducted at the Southwest National Primate Research Center. The animals were housed in individual cages for the duration of the dietary challenge in order to monitor individual patterns of food consumption. Standard health assessments, including blood chemistry and hematology profiles, were conducted on all animals before their assignment to the study. The animals had no significant history of health problems. The Institutional Animal Care and Use Committee of the Texas Biomedical Research Institute approved the study procedures. At baseline, all animals were fed the commercially available 5LE0 solid feed diet (LabDiet, PMI, St. Louis, MO). This diet ("chow") is high in complex carbohydrates and low in fat. Animals also had *ad libitum* access to water. Following an overnight fast, animals were sedated with ketamine (10 mg/Kg) before baseline body composition was assessed and blood samples were collected. We randomly assigned animals to receive one of the two diets for a period of 7 weeks, ten animals were assigned to a diet high in saturated fats, named the HSF diet, and eight animals were assigned to a diet high in unsaturated fats, the HUF diet. Both diets derive 45% of their calories from carbohydrates (mainly from simple carbohydrates), 15% of their calories from protein, and 40% from fat. Additionally, both groups were given a sweet drink containing simple carbohydrates *ad libitum*. Details of the experimental diets are given in **Table 1**. Blood samples were collected at baseline and at 3 and 7 weeks after initiation of the experimental diets.

Chemicals and antibodies

Reagents for particle isolation and quantification included BSA (Sigma, St. Louis, MO), 0.5 M EDTA solution (Sigma), and PBS solution (Invitrogen, Carlsbad, CA). The mixture of the reagents was filtered through 0.22 um membrane filter before each experiment. A flow cytometry size calibration kit was obtained from Life Technologies, Carlsbad, CA. Other materials included AccuCount blank particles

from Spherotech (#ACBP-50-10, Lake Forest, IL) and a Dynabeads magnetic sorting kit with pan mouse IgG, from Life Technologies. Mouse monoclonal antibodies included purified anti-human CD41 (BioLegend, San Diego, CA, clone HIP8) for platelet depletion, anti-human CD62E-FITC (R&D Systems, Minneapolis, MN, clone BBIG-E5), anti-human CD54-APC (BioLegend, clone HA58), anti-human CD106-PE (US Biological, Salem, MA, clone 5K26T), anti-human CD45-PerCP (BD Biosciences, clone D058-1283), anti-human CD41-Pacific blue (BioLegend, clone HIP8), anti-human CD105-APC (eBiosciences, San Diego, CA, clone SN6), anti-human CD31-APC/Cy7 (BioLegend, clone WM59). Isotypes for the above antibodies were IgG1-FITC (R&D Systems), IgG1-PE (BD Biosciences), IgG1-PerCP (BD Biosciences), IgG1-Pacific blue (BD Biosciences), IgG1-APC (BioLegend), and IgG1-APC/Cy7 (BioLegend). All antibodies used in this study have been either tested for their cross-reactivity to the baboon [12, 13] or confirmed as non-cross-reactive based on results from an NIH nonhuman primate reagent resource (www.nhpreagents.org).

Circulating particle determination

Our method of sample processing was modified from previous reports [5, 7, 15]. We collected whole blood samples in 10-ml EDTA tubes. Blood samples were centrifuged at 160 *g* for 10 minutes at room temperature within one hour after blood collection to harvest plasma. A 500-ul supernatant aliquot was transferred into a 5-ml polypropylene tube (BD Biosciences) that had been cooled on ice. To prepare platelet-poor plasma, we depleted the platelets with magnetic beads pre-coated with mouse anti-human CD41 monoclonal antibody, according to the manufacturer's instructions. All steps were performed on ice. One hundred ul of Dynabeads was added to the plasma and incubated on ice for 30 minutes with gentle tilting and rotation every 10 minutes. Three 100-ul aliquots of platelet-poor plasma were incubated, respectively, with (1) a mixture of 40 ul staining solution (1% BSA in PBS) containing 6 ul anti-human CD62-FITC, 7 ul anti-human CD54-APC, 5 ul anti-human CD106-PE, 5 ul anti-human CD41-Pac blue, 5 ul anti-human CD31-APC Cy7, 5 ul anti-human CD45-PerCP and 7 ul staining solution; (2) a mixture of 40 ul staining solution containing 5 ul anti-human CD31-FITC, 5 ul anti-human CD105-APC, 5 ul anti-human CD41-Pacific blue, 5 ul anti-human CD45-PerCP and 20 ul staining solution; and (3) a mixture of 40 ul staining solution containing all isotype controls. Before flow cytometry was performed, 860 ul of staining solution was added. We developed a flow cytometry procedure to measure the endothelial particles of different populations previously described [4, 7, 16]. The flow cytometer (Cyan ADP, Beckman-Coulter, CA) was calibrated and forward scattered light was set to include particles in diameter between 1-3 um in size using size calibration particles (Life Technologies). Samples were run at low speed to avoid flow disturbance for 10 minutes. Gating strategies were set to analyze populations that were negative for CD41 and CD45 and that were positive either for certain static (CD31 and CD105) or activated endothelium (CD106, CD54, and CD62E) markers. In each experiment, a tube of AccuCount blank particles (50 ul + 950 ul of filtered 1% BSA-PBS) was run for 10 minutes at the same speed as the test tubes. We calculated the absolute numbers using the formula: GE/AcE × Acperml/100, where GE is number of gated events, AcE is the number of Accucount particle events, Acperml is number of AccuCount particles per ml (calculated from the bottle label), and 100 is the quantity of plasma in ul. We coated magnetic beads with antibody against CD41, highly expressed on platelets, and used the beads to partially deplete platelets from the plasma in order to facilitate particle counting by flow cytometry.

Statistical analysis

Data were summarized with the median and first (Q1) and third (Q3) quartiles [Q1, Q3]. In the figures, the rectangles enclose the second and third quartiles, the horizontal line within the rectangles represents the median and the whiskers extend to the lesser of 1.5 times the standard deviation and the maximum or minimum values in original units. Due to skewness and zero values, preventing log transformation, the standard repeated measures parametric linear model was not applicable. Therefore, within group contrasts between time points were carried out with the non-parametric Wilcoxon signed rank test. Within-subject differences on paired measurements across time (week 0 versus week 3, week 3 versus week 7, week 0 versus week 7) by group (HSF and HUF) were calculated. All statistical testing was two

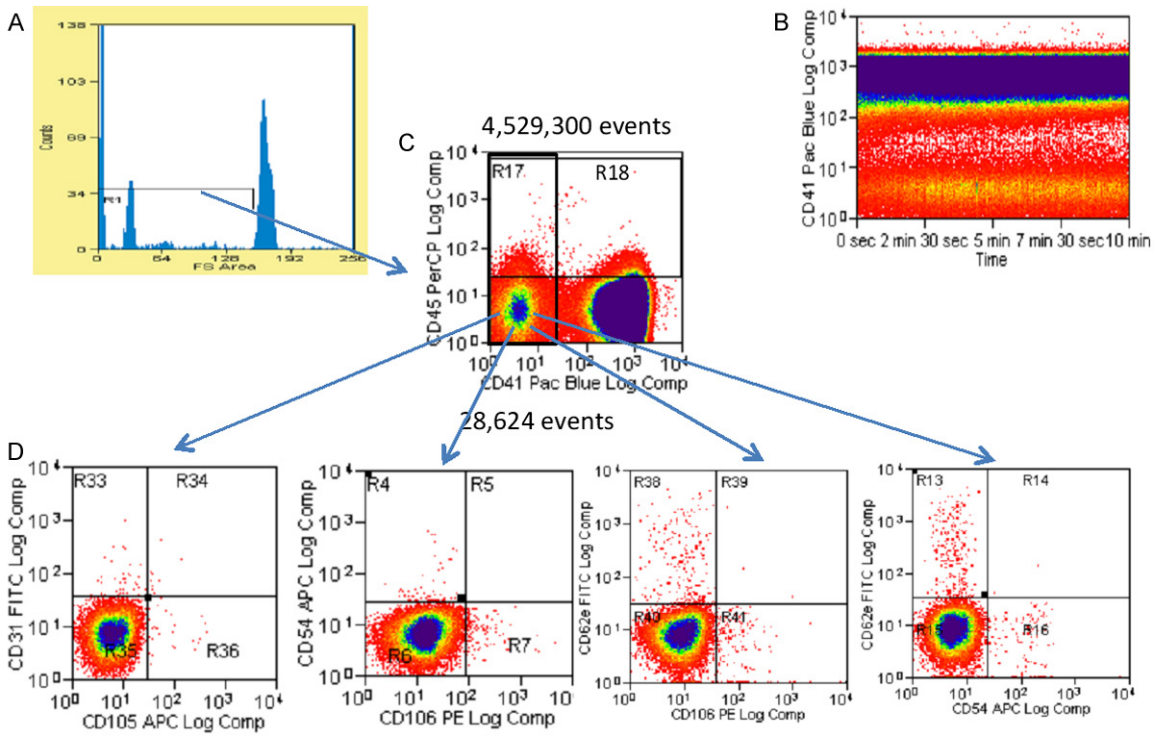

Figure 1. Diagrams of flow cytometry determination of particles in plasma. In order to establish a standardized protocol for accurate determination of endothelial particles in plasma, we defined the conditions that allowed us to acquire consistent and reliable data. A: Histogram analysis of the forward scatter of polystyrene nonfluorescent microspheres with known sizes; the two peaks represent beads with different diameters of 1 and 4 um, respectively, which were used for size-position. B: Demonstration of steady flow of CD41+ particles in plasma samples during 10 minutes while the samples were processed in the flow cytometer. C: Flow cytometry plots showing the distribution of events acquired at size positions between 1 and 4 um. A representative count is shown. Among 4,529,300 events collected, there were 28,624 particles that are CD45-/CD41-, which were clearly separated from CD45-/CD41+. D: Analysis of particles with different phenotypic markers (four panels are shown). By gating at CD45-/CD41- regions, we could identify different antigenic markers with fluorescent conjugate combinations. For example, R33 in the first panel represents the specific population of CD31+/CD41-/CD45- particles while R38 in the third panel represents CD62E+/CD41-/CD45-.

sided with a significance level of 5%. SAS Version 9.3 for Windows (SAS Institute, Cary, North Carolina) and R Version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) were used throughout.

Results

Both diets elicited significant elevations of LDL-cholesterol and triglycerol and, in both diets, levels of plasma C-reactive protein exhibited a steady increase, proinflammatory cytokines such as MCP-1 and IL-8 were released into circulation in physiologically significant quantities, and inflammatory monocytes peaked after 3 weeks. Therefore, both diets appeared proinflammatory with atherogenic properties.

A representative plot of particle detection based on coated magnetic beads is shown in **Figure 1**. We acquired approximately 4,529,300 events in diameters ranging between 1 and 3 um (**Figure 1A**) with a steady reading at 10 minutes (**Figure 1B**). A second gate was set as CD41- and CD45- (**Figure 1C**); endothelial particles with different antigenic markers were detected using this gating strategy as illustrated in the four diagrams (**Figure 1D**). A 10-minute counting was enough to guarantee more than 20,000 events in the CD41-/CD45- population. The histograms shown here suggest that this methodology enables us to separate and to count circulating particles. We used CD31 and CD105 as phenotypic markers for detecting cellular structural damage or integrity under static condition, and CD62E, CD54, and CD106 as markers of endothelial activation [12, 17, 18]. In this study, our results appear to demonstrate that the changes, both increases and

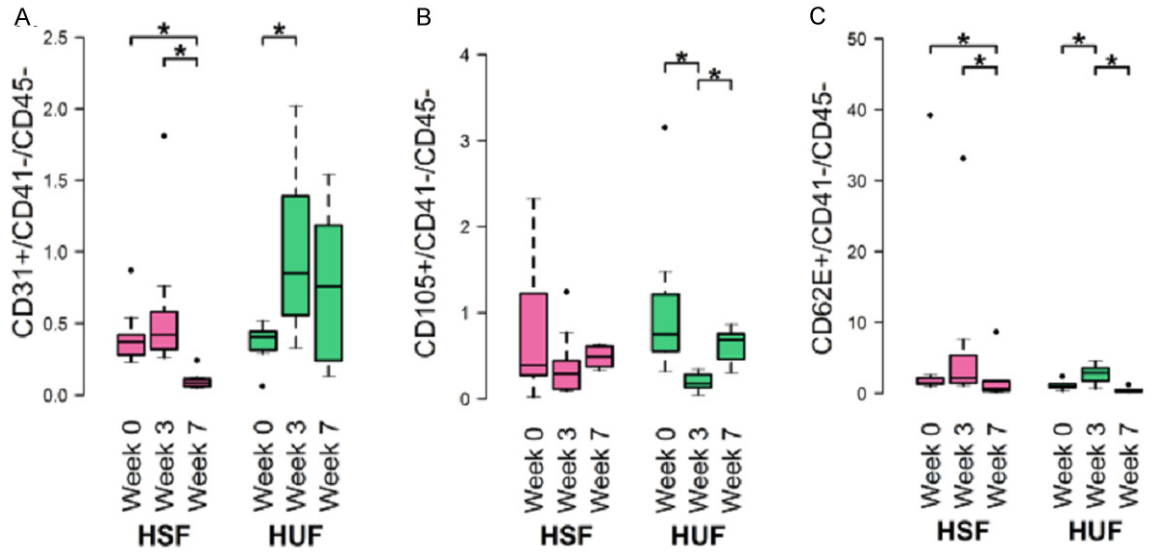

Figure 2. Percentages of CD31+, CD105+ and CD62E+ circulating endothelial particles in baboons subjected to 7-week HSF challenge or 7-week HUF diet challenge. Plasma samples were collected at indicated time points and stained for different markers representing endothelial origin. Percent changes of positive particles are shown for different subpopulations of particles derived from endothelial cells. Wilcoxon signed rank test was used to compare within-subject differences on paired measurement across time (week 0 versus week 3, week 3 versus week 7, week 0 versus week 7) by groups (HSF and HUF). Statistical significance (*) was set at $p < 0.05$. A: CD31+/CD41-/CD45-. B: CD105+/CD41-/CD45-. C: CD62E+/CD41-/CD45-.

decreases, in percentages and absolute counts of particles with specific cell surface markers are time-dependent.

We studied the phenotypic changes of the endothelial particles as a percentage of all particles. Three populations of particles (CD31+/CD41-/CD45-, CD105+/CD41-/CD45-, CD62E+/CD41-/CD45-, abbreviated as CD31+, CD105+, CD62E+, respectively) were considered and are described in **Figure 2**. Results were obtained at baseline (week 0), and at 3 and 7 weeks after dietary challenge.

At week 3, by comparison with week 0, the percentage of CD31+ particles in ten animals fed HSF diet had increased from a mean of 0.37 at baseline to 0.42 (**Figure 2A**), but the change was not statistically significant ($p = 0.54$, $n = 10$). The percentage of CD31+ particles in eight animals fed HUF diet demonstrated significant elevation from 0.40 at baseline to 0.85 ($p = 0.02$, $n = 8$). Similarly, the percentage of CD62E+ particles was increased but not significantly, in response to HSF diet ($p = 0.38$, $n = 10$), whereas the increase in response to HUF diet was significant ($p = 0.01$, $n = 8$, **Figure 2C**). The percentage of CD105+ particles was reduced at week 3 without significance ($p = 0.28$, $n = 10$) in response to HSF diet as shown in **Figure 2B**; but the decrease was significant in animals fed HUF diet 0 ($p=0.02$, $n=8$).

At week 7, the percentages of CD31+ and CD62E+ particles were lower by comparison with week 0 ($p < 0.05$, $n = 10$) in the HSF diet group, but not in the HUF diet group (**Figure 2A**, **2C**). Comparison of results from weeks 3 and 7 revealed significant reductions in percentages of CD31+ and CD62E+ particles in response to HSF diet in animals fed the HSF diet, and in CD62E+ particles in animals fed the HUF diet.

No significant elevations were observed in percentage of CD105+ particles in animals fed the HSF diet, but median percentage of CD105+ particles was significantly lower at week 3 in comparisons to week 0 and week 7 in the HUF diet group.

In addition to measuring phenotypic changes of the endothelial particles as a percentage of all particles, we measured absolute counts in log-units as shown in **Figure 3**. In response to HSF diet, we found significant decreases in the number of CD31+ particles at week 7 relative to week 0 and week 3 ($p < 0.01$, **Figure 3A**) but we found no significant changes, either increas-

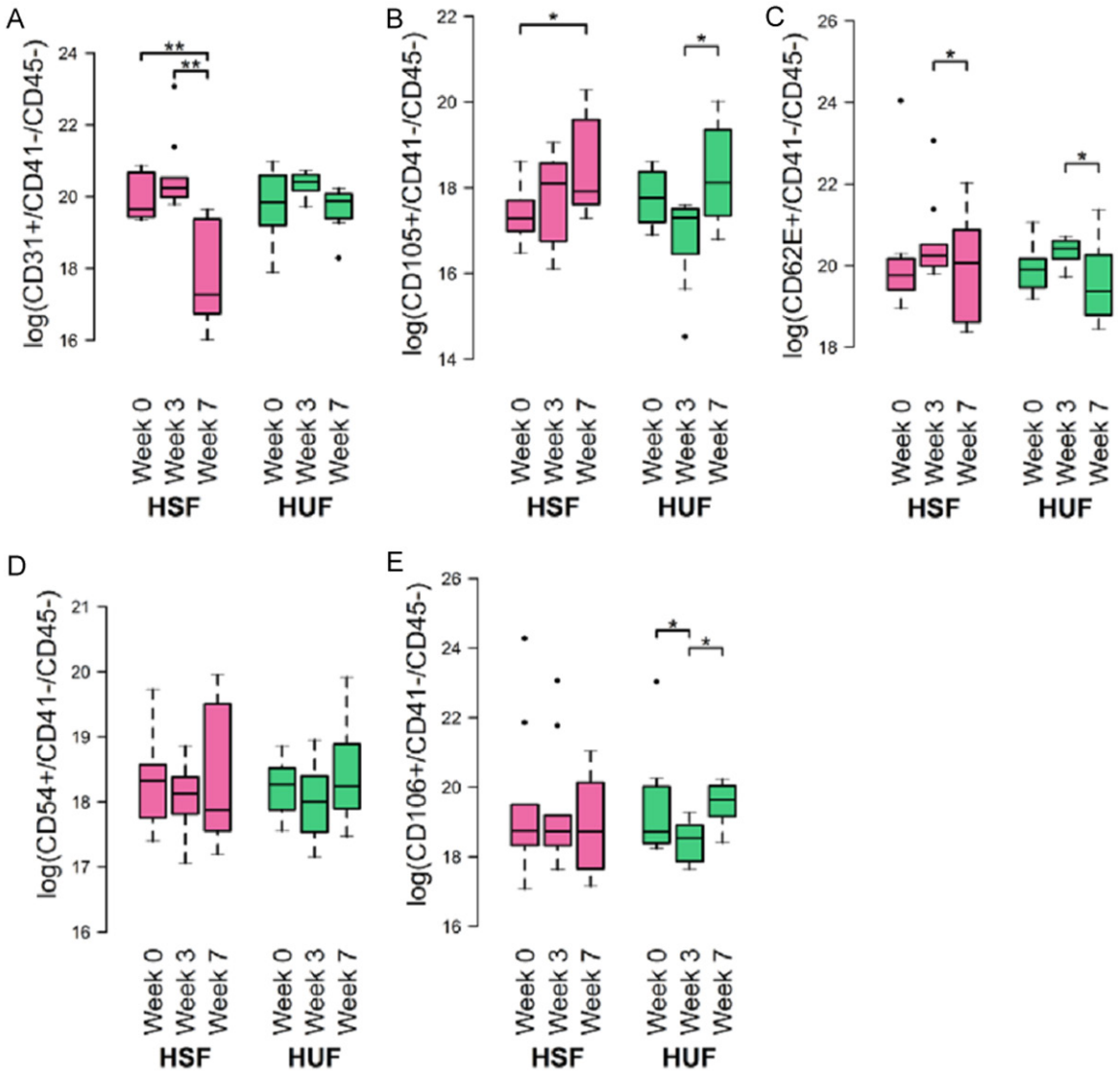

Figure 3. Absolute counts (× 10^6 particles per 100 ul plasma) of circulating endothelial particles in baboons subjected to a 7-week HSF challenge or 7-week HUF challenge. The absolute counts of circulating particles were calculated based on the total gated events using AccuCount particles (Spherotech) as an internal comparison, as detailed in Materials and Methods. A: CD31+/CD41-/CD45-. B: CD105+/CD41-/CD45-. C: CD62E+/CD41-/CD45-. D: CD54+/CD41/-/CD45-. E: CD106+/CD41-/CD45-.

es or decreases, in response to the HUF diet. The median numbers of CD62E+ particles were higher at week 3 than week 0 and week 7 in response to both diets (**Figure 3C**), consistent with the alterations presented in **Figure 2A** and **Figure 2C**. In both diets, decreases in the median numbers of CD62E+ particles between weeks 3 and 7 were significant. The median number of CD105+ particles were elevated in response to the HSF diet between week 0 and week 7 (**Figure 3B**). This pattern was not seen in response to the HUF diet (**Figure 3B**); the median number of CD105+ particles was decreased at week 3 and increased at week 7. Similarly, the number of CD106+ particles was significantly lower at week 3 relative to week 0 and week 7 in animals fed HUF diet (**Figure 3E**), but not in those fed HSF diet. The number of CD54+ particles did not demonstrate significant alterations in response to either diet (**Figure 3D**).

Discussion

When endothelial cells are stressed, cellular membrane bleb and form vesicles that are shed to the blood stream. The fragments derived from the cells can be classified as small

(with a diameter between 0.1 and 1.0 um) or large (with a diameter > 1.0 um). Small circulating cellular microparticles have been thoroughly investigated as biomarkers of vascular integrity for decades [19-22], however, less is known about altered circulating levels of large particles with diameters between 1 and 3 um in response to vascular damage. In this study, we focused on particles with diameters ranging between 1 and 3 um, because we had already observed that endothelial cells isolated from baboon femoral arteries give rise to large particles when treated with proinflammatory cytokines or modified LDL derivatives (data not published). The current study we tested the hypothesis that a 7-week dietary challenge results in endothelial particle shedding into the systemic circulation.

Although several methods have been used to detect circulating endothelial particles, none are well-recognized and accepted [23, 24]. Many protocols measured cellular particles in plasma rendered platelet-poor by centrifugation at a variety of speeds and under a variety of conditions. Centrifugation may cause partial loss of circulating particles from the plasma, especially particles of diameter 1 um or more, leading us to speculate that centrifugation might lead to imprecise and inconsistent results [23]. If the samples are not centrifuged, the overlap in size of platelets and particles impedes the detection of particles by flow cytometry. Therefore, we modified the traditional protocol by removing most of the platelets through magnetic bead depletion; consequently, we increased the capacity of the system to detect and quantify particles by flow cytometry. Our method has three advantages: (1) particles with their distinct signatures can be measured in their native form in plasma samples without loss due to processing, (2) particles can be defined precisely with the use of multiple antigenic markers and counted with the use of specific internal size controls, and (3) cellular particles are now distinguished clearly from platelets, facilitating the data acquisition and processing. This improvement facilitates accurate measurement for circulating particles in plasma and may become a platform for standardized particle determination [21, 23, 24].

With methodological improvement, we were able to observe vascular endothelial damage at early stages due to atherogenic diets. As early as 3 weeks after dietary challenge, animals fed HSF diet exhibited signs of endothelial damage (CD31+ particles in **Figures 2A**, **3A**) and activation (CD62E+ particles in **Figures 2C**, **3C**), but the median values were not significantly different from the baseline medians; the small sample size combined with large inter-individual variations prevented detailed study of these patterns. Between weeks 0 and 3, animals fed HUF diet demonstrated significant elevations in percentages of CD31+ particles (**Figure 2A**) and CD62E+ particles (**Figure 2C**), although the increases in the median absolute counts of these particles did not reach statistical significance (**Figure 3A** and **3C**). CD105+ particles, indicators of structural integrity, were progressively and significantly elevated in response to HSF diet (**Figure 3B**).

This study provides a research tool that detects the early vascular damage due to a variety of experimental manipulation. Although optical and ultrasonic technologies have been increasingly applied in tracking acute and chronic injury to vasculature, changes in endothelial particles may mirror those pathological alterations at the cellular level and therefore may reveal the earliest events in regard to vascular responses to diet-induced injury. Because flow cytometry enables multiple markers to be investigated simultaneously, it is possible to track different particles during treatment and to document the sequence of pathogenic processes that target endothelial cells. Our results indicate that replacement of dietary saturated fat with unsaturated fat in the presence of a high level of simple carbohydrates did not materially alter the patterns of changes in circulating endothelial particles. The dogma that unsaturated fats may be less damaging to arteries than saturated fats is now open to question since there is increasing evidence that polyunsaturated fatty acids may induce changes in membrane lipid composition that enhance oxidative stress and induce apoptosis [25-27]. In baboons fed HUF diet, the median percentage of CD31+ particles increased to a greater extent than in baboons fed HSF diet during the dietary challenge, suggesting that arterial damage in response to unsaturated fat increased in response to saturated fat (**Figure 2A**); moreover, the increase from week 0 to week 3 was statistically significant in response to HUF diet only. This result suggests that HUF diet actually caused more endothelial damage

than HSF diet during between weeks 0 and 3. Changes in the median number of CD62E+ particles were similar in response to both diets. It appears that both diets activated the endothelium to the same degree, although HUF may have had a more potent destructive effect than HSF.

An intriguing finding is that the levels of some particle types decrease significantly in baboons after 3 weeks or 7 weeks of dietary challenge, as has been seen in patients with acute inflammation or multiple organ dysfunctions [28]. This observation is consistent with the belief that the decreased particle levels in plasma could result from adhesion to target tissues and that particles derived from cells may exert biological effects. Mounting data from recent years have supported this belief by documenting that the fragments or particles from damaged cells are able to bind to Toll-like receptors, IL-1 receptors and RAGE receptors and to elicit proinflammatory responses [29], involving the production of cytokines and the recruitment of inflammatory cells. This type of inflammation is well known to play a key role in atherosclerosis. Moreover, exogenous administration of endothelial particles is associated with systemic inflammation and other disorders [30]. Endothelial particles of small size can be vehicles that mediate intercellular communication, regulating a number of physiological and pathological processes [3, 31]. The consistent decreases in both the percentage and numbers of particles in our study in response to diets at week 7 may reflect important and perhaps complex mechanisms affecting diet-induced vascular alterations. The study of such hypothetical mechanisms is important but is beyond the scope of this study.

The results of this study have direct implications for the clinical investigations. The combination of the animal model and our methodological improvement enhances the potential for particles to be developed as biomarkers for translational medicine. The baboon, being a large nonhuman primate, shares many physiological characteristics with human beings and is an important and relevant model for human atherogenesis suggesting that the results obtained here are likely to reflect the scenarios that occur in human subjects. An important feature of this study was the longitudinal design, which enabled us to detect the changes that reflect the time course of pathological events induced by diet. We documented that particles increase and decrease over time, depending on the population, providing experimental evidence that may explain some of the variation among previous reports. The dynamic changes provide a unique set of biomarkers of endothelial damage with stage-specific characteristics. Our results may enable valid interpretations of the conflicting reports derived from clinical investigations based on population studies or from rodent models.

The principal limitations of this study were the small numbers of animals and the few observation time points. Both limitations stem from the high cost and management of the primate model. While statistical significance may have been reached in more time point comparisons if the number of animals had been larger, the fact that statistical significance was reached at all suggests that the response to these diets is robust. Even with these limitations, the results of the percentage analyses and counts of endothelial-derived particles observed during the dietary challenge encourage further investigation, with an ultimate goal of establishing an accurate and sensitive tool that can enhance our understanding of how diets affect blood vessels and impact the development of the cardiovascular diseases.

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performed flow cytometry, and acquired data. Dr. Vida Hodara had also a major contribution to manuscript writing. Dr. Qinghe Meng provided data processing methods. Joel E. Michalek performed final statistical analysis. Karen Rice coordinated the animal dietary treatment and sample collection. Drs. Antony G. Comuzzie and John L. VandeBerg developed overall research plan and oversight the study. Drs. John L. VandeBerg and Joel Michalek contributed to manuscript writing and revising.

Disclosure of conflict of interest

None of the authors has financial conflict of interest to be disclosed.

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