Original Article

Platelet-related cytokines among normal body mass index, overweight, and obese Malaysians

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Background and Objectives: Recent studies have reported that obesity is associated with platelet activation and systemic inflammation. Malaysia has the highest prevalence of obesity, hence, this research is performed to evaluate the development of low-grade inflammation and platelet activation, measured using soluble CD40 ligand (sCD40L) and soluble P-selectin (sP-sel), and to determine their association with obesity. In addition, we assessed the mean platelet volume (MPV) and platelet count (PLT), which are novel parameters consistently associated with obesity. **Methods and Study Design:** A cross-sectional study was conducted on 112 healthy men and women from 3 main ethnic group (Malay, Chinese, and Indian) who were aged 18–60 years. The participants were categorized into normal body mass index (BMI), overweight and obese groups according to WHO criteria for BMI in Asian populations (18.5 kg/m^2<BMI<35 kg/m^2). Waist circumference (WC) was also measured and included in the analysis. **Results:** MPV, sCD40L, and sP-sel differed significantly among the normal BMI, overweight, and obese groups (p<0.05). Contrastingly, the PLT did not vary significantly among the 3 groups. In addition, sP-sel levels correlated significantly with BMI (r=0.36, p=0.001) and WC (r=0.25, p=0.007) and MPV correlated significantly with BMI (r=0.2, p=0.001) and WC (r=0.2, p=0.003). **Conclusions:** Higher MPV and sP-sel levels in the obese participants than in the overweight and normal BMI participants indicated potentially higher activation of platelets in people with obesity. Moreover, we observed higher sCD40L levels in obese participants than in the overweight and normal BMI participants, suggesting a proinflammatory state in obese individuals.

Key Words: body mass index, soluble CD40 ligand, mean platelet volume, soluble P-selectin, waist circumference

INTRODUCTION

Malaysia is considered a multi-ethnic country, with the highest prevalence of obesity among Asian countries.[1] Approximately 50% and 45% of Malaysian women and men, respectively, are overweight or obese.[2] Obesity is considered a multifactorial and complex disease. As the immune and metabolic systems are closely associated, excessive consumption and storage of nutrients can overload signaling networks, leading to the alteration of these systems.[3] Thus, cytokines, hormones, transcription factors, bioactive lipids, and signaling proteins have both immune and metabolic roles.[4]

Soluble CD40 ligand (sCD40L), has recently been indicated as a novel inflammatory marker. sCD40L is shed from activated platelets, and approximately 95% of circulating sCD40L is derived from inactivated platelets.[5] The CD40L receptor, CD40 is considered an integral membrane protein present in various cells such as monocytes, fibroblasts, and endothelial and vascular smooth muscle cells.[6] sCD40L levels are altered under certain conditions, such as cardiovascular disease (CVD),[7] and are elevated in diabetes[8] and obesity.[9] Under chronic inflammatory conditions, T cells have been reported to be polarized toward the Th1 subset by CD40 interactions.[10] This action has a damaging effect on this dyad in obesity. Considering obesity as a proinflammatory state, the association between sCD40L and obesity is an active research area.

Soluble P-selectin (sP-sel) is a marker of interest because it can regulate interactions between the endothelium and blood cells.[11] sP-sel (CD62) is a transmembrane protein mainly synthesized by platelets and endothelial cells. P-selectin (P-sel) is stored in α-granules in inactivated platelets and is expressed on the plasma membrane after cell stimulation, typically during inflammatory responses.[12] sP-sel is the soluble form of P-sel and is mainly derived from activated platelets. Therefore, it can be

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considered a circulating marker of platelet activation.[13] Studies on the effect of obesity on sP-sel levels have reported controversial outcomes. One study[14] demonstrated a direct association between the BMI and considerably elevated sP-sel levels in obese participants, whereas Vázquez et al[15] reported no notable differences in sP-sel levels between obese and normal BMI participants.

Mean platelet volume (MPV) was investigated because it is closely associated with *in vivo* platelet activation; thus, platelet activation is a link in the pathophysiology of diseases associated with thrombosis and inflammation.[16] Moreover, several studies have reported that the association between MPV and obesity is a conventional cardiovascular (CV) risk factor, suggesting a possible mechanism underlying an increase in CV risk.[17,18]

Direct studies on and related findings in the Malaysian population are lacking, therefore, we evaluated sCD40L and sP-sel levels associated with platelet activation and inflammation in men and women with varying body mass indices (BMIs). This study provides a data for future research on obesity, particularly in Malaysia's multi-ethnic population.

MATERIALS AND METHODS

Study design and location

This cross-sectional study was conducted in the Haematology Laboratory and Dietetic Clinic at the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (FMHS, UPM), from August 2014 to November 2015. Ethical approval was obtained from the Ethics Committee for Research involving Human Subjects of UPM [UPM/TNCPI/RMC/1.418.1(JKEUPM)/F2].

Participants

This study analyzed Malaysian citizens aged 18–60 years. They were equally categorized into a control group (normal BMI, 18.5-22.9 kg/m^2) and case group (overweight and obese; BMI, 23-35 kg/m^2). All study participants were disease-free (healthy) and were recruited from the staff and students of UPM by distributing flyers and an email advertisement. The study was verbally explained to the participants, and a participant information sheet was provided to them before they signed a standardized consent form as evidence of written approval at the Dietetic Clinic. A pro forma form was used to collect participant data on sociodemographic characteristics, family history, medical history, current medications, and risk factors for inflammation (e.g. smoking, allergies, and alcohol consumption).

Anthropometric measurements

Two essential indices for measuring the BMI were recorded at the Dietetic Clinic: weight (using a weighing scale) and height (using a measuring tape). Furthermore, waist circumference (WC) was measured at the natural indentation between the 10th rib and iliac crest (minimum waist). The participants were categorized into the control and case groups according to the World Health Organization criteria for BMI in Asian populations.

Laboratory investigations

A phlebotomist at the Haematology Laboratory drew 10 mL of venous blood from the antecubital fossa and stored it in appropriately labeled tubes; 3 mL of the blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube for full blood count (FBC) analysis, and the remaining 7 mL was collected in plain tubes for biochemical testing by using the enzyme-linked immunosorbent assay (ELISA) method.

A sample of peripheral blood for the FBC analysis was examined using a Sysmex KX-21 within 4 hours of collection, to determine the MPV and platelet count (PLT) in 1 µL of whole blood. The Sysmex KX-21 is an automated, multiparameter blood cell counter for in vitro diagnosis. It has flow cells, photometers, and apertures that analyze different blood elements. Laser eye sensors identify and enumerate the number of cells passing through the aperture. Calibration and third-party quality control were performed before each run to ensure the accuracy and precision of the results.

To obtain serum for the ELISA measurements of sCD40L and sP-sel, the blood samples were allowed to clot for 15–30 minutes, and the tubes were centrifuged at 2200×g for 10 minutes. The serum was subsequently aliquoted into vials and stored at −20°C until further batch analysis. The samples were thawed only once. All samples, controls, and standards were tested in duplicate. Before starting the test procedure, the reagents and samples were diluted according to the values stated in each protocol. The samples were manually analyzed using commercial ELISA kits (BD Biosciences for sCD40L and sP-sel). The absorbance of each microwell was read on a spectrophotometer at 450nm by using an automated ELISA plate reader (Chromate ELISA Reader) with the suggested filters. According to the manufacturer, the calculated overall intra-assay coefficients of variation for sCD40L and sP-selectin are 6.8% and 7.8%, with a sensitivity of 0.062 and 0.20 ng/mL, respectively.

Statistical analysis

Statistical calculations were performed using SPSS version 22 for Windows (IBM Inc., USA). Descriptive statistics, including frequency tables, means, and standard deviations were calculated. Before statistical testing, all variables were subjected to a normality test based on skewness and kurtosis for continuous variables. Inferential statistics, namely the independent t test and one-way analysis of variance (ANOVA), were used for between-groups comparisons, and the Pearson correlation method was applied to evaluate the relationships among the research variables. In all analyses, $p<0.05$ (95% confidence interval) was considered statistically significant.

RESULTS

A total of 112 participants were categorized into two groups: the control (n=56) and case (n=56) groups. Most participants were Malays (57% cases and 52% controls), followed by Chinese (16% cases and 32% controls) and Indians (27% cases and 16% controls). Overall, 54.5% of the participants were women (44.6% cases and 64.2% controls), and 45.5% of them were men (55.4% cases and 35.8% controls). Most participants were allergy-free (83%) and did not consume alcohol (87.5%), while all participants were non-smokers. The means and standard

Table 1. Basic characteristics of the participants based on their BMI[‡]

Parameter	Mean±SD			*p* value[†]
	Normal (n=56)	Overweight (n=28)	Obese (n=28)	
Age (years)	28.6±7.0	28.1±8.5	28.0±8.0	0.9
Weight (kg)	53.1±6.7	67.6±8.3	84.7±17.5	0.001**
Height (cm)	161 ±8.5	167 ±10.2	165±10.7	0.01
BMI (kg/m^2)	20.4±1.2	24.0±0.8	30.8±5.3	0.001**
WC (cm)	71.3±10.5	84.0±8.6	96.6±10.0	0.001**
MPV (fL)	8.7±1.5	9.5±0.7	9.5±0.8	0.012*
PLT (×10^3/µL)	280±71.0	265±61.6	297±70.0	0.236
sCD40L (ng/mL)	5.2±5.8	6.4±7.1	10.1±8.1	0.045*
sP-selectin (ng/mL)	130±70.2	138±60.9	188±57.3	0.005**

MPV: mean platelet volume; PLT: platelet count; sCD40L: soluble CD40 ligand; sP-selectin: soluble P-selectin; SD: standard deviation; BMI: body mass index; WC: waist circumference.
[†]One-way ANOVA: Post hoc, followed by multiple mean comparison; *p<0.05, **p<0.01; n (number of individuals in each group), [‡]Normal: BMI, <23 kg/m^2; overweight: BMI, 23–25 kg/m^2; obese: BMI, >25 kg/m^2.

deviations of the laboratory parameters and anthropometric characteristics of the 112 participants are summarized in Table 1. Significant differences were observed in the mean weight, BMI, and WC between the case and control groups (p<0.05). The obese and normal BMI groups, respectively, had the highest and lowest mean weight (84.7±17.5 vs 53.1±6.7, p=0.001), BMI (30.8±5.3 vs 20.4±1.2, p=0.001), and WC (96.6±10.0 vs 71.3±10.5, p=0.001). As shown in Table 1, the obese and normal BMI groups, respectively, had the highest and lowest MPV (9.5±0.8 vs 8.7±1.5, p=0.012), sCD40L (10.1±8.1 vs 5.2±5.8, p=0.045), and sP-sel (188±57.3 vs 130±70.2, p=0.005), and no significant difference was observed in the mean PLT between these groups.

One-way ANOVA, followed by the Duncan post hoc test, were both used for a pairwise comparison of biomarker levels, because there were more than two groups per biomarker (normal BMI, overweight, and obese). The MPV was significantly higher in obese and overweight participants than in normal BMI participants (p=0.012). However, no significant difference was observed in the mean of the MPVs between the obese and overweight participants (Figure 1). The mean sCD40L levels in obese participants differed significantly from those in the overweight and normal BMI participants (p=0.045, however the difference between the overweight and normal BMI

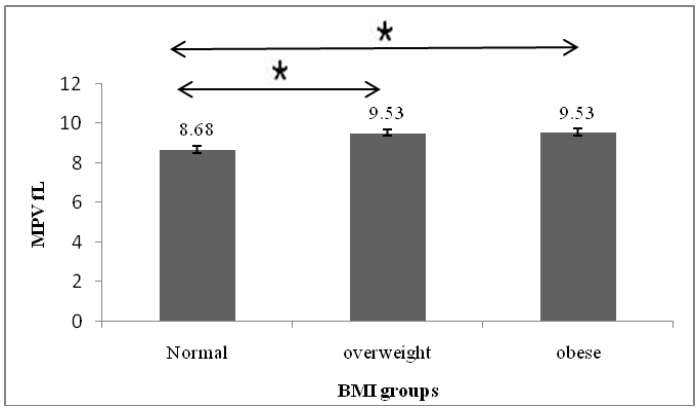

Figure 1. MPV values in the BMI-stratified groups.

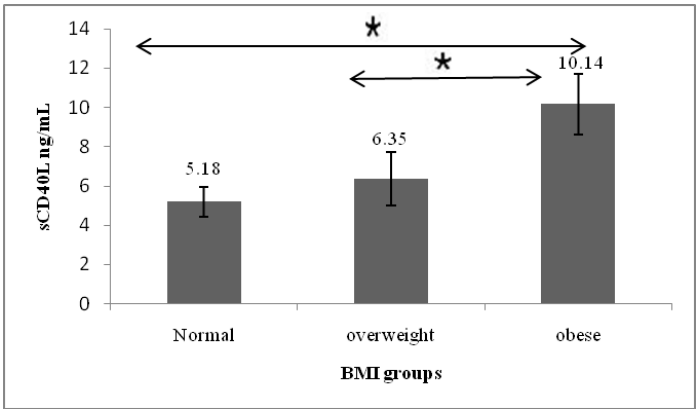

Figure 2. sCD40L levels in the BMI-stratified groups.

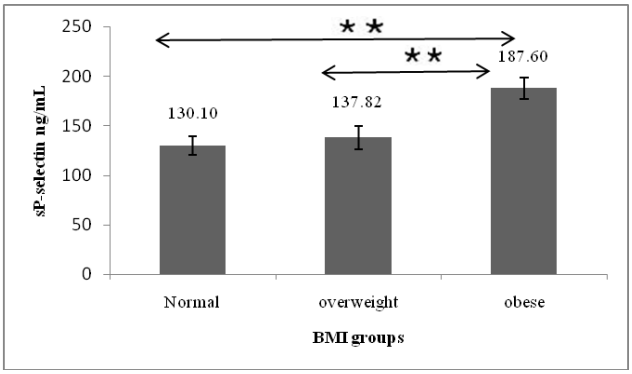

Figure 3. sP-sel levels in the BMI-stratified groups.

Table 2. Pearson correlation test of the association of the laboratory parameters with WC and BMI

Laboratory parameters	BMI		WC	
	r	*p*	r	*p*
MPV (fL)	0.20	0.001**	0.28	0.003**
PLT (×10^3/μL)	0.13	0.18	0.04	0.631
sP-selectin (ng/mL)	0.36	0.001**	0.25	0.007**
sCD40L (ng/mL)	0.24	0.01*	0.13	0.153

MPV: mean platelet volume; PLT: platelet count; sCD40L: soluble CD40 ligand; sP-selectin: soluble P-selectin; BMI: body mass index; WC: waist circumference.
r: Pearson product-moment correlation coefficient.
*p<0.05, **p<0.01.

Table 3. Laboratory parameters (PLT, MPV, and platelet-related cytokines) between ~~the~~ men and women in the normal BMI, overweight, and obese groups‡

Parameters	Groups	Men	Women	p†
MPV (fL)	Normal	9.12±0.67	9.02±0.76	0.402
	Overweight	0.75±9.43	9.56±0.75	0.897
	Obese	0.82±9.66	9.58 ±0.86	0.765
PLT(×10^3/μL)	Normal	278±75.5	282±69.5	0.858
	Overweight	238±44.5	314±59.7	0.005**
	Obese	292±64.0	301±75.9	0.710
sCD40L(ng/mL)	Normal	3.36±3.27	6.19±6.60	0.149
	Overweight	3.92±3.14	10.7±9.99	0.02*
	Obese	7.46±6.88	12.5±8.61	0.201
sP-selectin(ng/mL)	Normal	110±48.5	141±78.2	0.098
	Overweight	142±58.7	131±67.4	0.415
	Obese	160±64.9	212±37.6	0.187

sCD40L: soluble CD40 ligand; sP-selectin: soluble P-selectin; PLT: platelet count; MPV: mean platelet volume.
†One-way ANOVA, mean comparison based on the Bonferroni test.
‡Normal: BMI <23 kg/m^2; overweight: BMI, 23–25 kg/m^2; obese: BMI, >25 kg/m^2.
*p<0.05, **p<0.01.

participants was non significant (Figure 2). Similarly, sP-sel levels were significantly higher in the obese group than in the overweight and normal BMI groups (p=0.005). However, the difference in sP-sel levels between the overweight and normal BMI groups was not statistically different (Figure 3).

In all three groups, significant but weak positive correlations were observed between sP-sel levels and BMI (r=0.36, p=0.001) and WC (r=0.25, p=0.007), between MPV and BMI (r=0.2, p=0.001) and WC (r=0.2, p=0.003), and between sCD40L levels and BMI (r=0.2, p=0.01; Table 2).

Table 3 shows a comparison of the laboratory parameters (PLT, MPV, and platelet-related cytokines) between the men and women in the normal BMI, overweight, and obese groups. In the overweight group, PLT (314±59.7 vs 238±44.5, p=0.005) and sCD40L levels (10.7±9.99 vs 3.92±3.14, p=0.02) were significantly higher in the women than in the men (p<0.05). MPV and sP-sel did not differ significantly between the men and women, as stratified by BMI.

DISCUSSION

Scant knowledge is available about the marked changes in sCD40L levels in obesity. In the present study, sCD40L levels were significantly higher in the obese participants than in the overweight and normal BMI participants. This finding accords with previous studies that

have reported increased sCD40L levels in individuals with central obesity, and decreased sCD40L levels in those with reduced BMI. Accordingly, obesity may contribute to sCD40L overexpression.[19,20] Another study by IT Unek et al, reported that the amount of sCD40L increased remarkably in obese individuals (BMI >30 kg/m^2) compared with non-obese individuals (BMI <25 kg/m^2), while no notable differences were observed between the obese and overweight (BMI 25-29.9 kg/m^2) individuals,[5] probably because of the BMI classification, which is typically lower in Asian populations.[21] In our study, the participants who were classified as overweight (23 kg/m^2<BMI<25 kg/m^2) according to the Asian classification system would be considered to have a normal BMI in non-Asian populations.

In obese humans and animals, T cells tend to gather in adipose tissue[22] and participate in the onset of adipose tissue inflammation.[9] The adipose tissue synthesizes and secretes several cytokines, whose levels directly correlate with the degree of obesity. Considering that CD40L is mostly generated by activated CD4+ T cells and platelets,[23] elevated sCD40L levels in obese individuals may explain the relationships among obesity, inflammation, and atherosclerosis.[24] Furthermore, Desideri et al reported that, in adults, sCD40L levels and lipid peroxidation have a higher correlation than do sCD40L levels and BMI.[25] Similarly, our findings revealed a positive correlation between sCD40L levels and BMI. However, we did not examine lipid peroxidation, a marker of oxidative injury. Elevated oxidative stress is widely known in obesity, which might be associated with dysregulated adipocytokine production.[26] Circulating sCD40L is supposedly derived mainly from platelets. As previously reported, obesity is related to lipid peroxidation and platelet activation.[27] Thus, activated platelets are the main source of increased sCD40L levels in obesity. Nevertheless, the probable mechanism for the expression of sCD40L by activated platelets is not completely understood.

In the present study, significant differences in cytokine profiles were observed between the men and women in the overweight group. The women had higher sCD40L levels than the men, with obese women and normal BMI men displaying the highest and lowest sCD40L levels, respectively. Angelico et al reported higher sCD40L levels in female patients with metabolic syndrome than in their male counterparts.[28] Scarce data are available regarding the relationship between sCD40L and gender in obesity. The mechanism for increased sCD40L levels in healthy overweight and obese women as an indicator of inflammation has not yet been elucidated, however genetic factors might be a contributing factor.[29] The data from the present study can lay a foundation for future studies to demonstrate that high sCD40L levels in obese women might expose them to a higher risk of CV events, compared with those in obese men. Accordingly, in the future, sCD40L can be proposed as a biomarker of cardiovascular disease (CVD), either alone or in combination with other markers. The non-significant correlation between sCD40L levels and WC suggests that the increased sCD40L concentration in obese individuals is independent of visceral fat.

This study confirmed a significant relationship between sP-sel and obesity. Similar studies by Genc et al and De Pergola et al have evaluated sP-sel as a fundamental marker of platelet activation.[30,31] Genc et al reported similar sP-sel levels in prediabetic and nondiabetic individuals. However, De Pergola et al noted significantly higher sP-sel levels in obese individuals than in those with a normal BMI.

Considering the role of sP-sel in platelet activation,[30] increased levels of this marker imply a higher aggregating activity of platelets in obesity.[32] The significantly increased sP-sel levels in the obese participants in the present study may be due to obesity-induced endothelial activation or increased shedding of cell surface P-selectin, leading to increased sP-sel.[33] This study demonstrated that conventional measures of obesity (BMI and WC) are related to adhesion molecule levels. Both BMI and WC correlated significantly with sP-sel, suggesting that cytokines, arising in part from visceral adipose tissue, partially account for endothelial activation and dysfunction in obese individuals, particularly in those with visceral obesity.[11] However, a cross-sectional study by De Pergola et al demonstrated no correlation between WC and sP-sel levels.[30] Moreover, in the present study, the obese women and men had the highest and lowest sP-sel levels, respectively. These observations suggest that female hormones may play a significant role in sP-sel levels.[34]

Moreover, the present results support the hypothesis that obese and overweight individuals have a higher MPV than normal BMI participants. These findings accord with those reported by Shah et al, who showed significantly higher MPV in individuals with abdominal obesity.[35] As MPV is a factor for platelet activation and size, one mechanism for higher MPV levels in obese individuals may involve damaged endothelial cells and the induction of atherosclerotic processes by obesity, which are crucial interactions between activated endothelial cells and platelets. Thus, increased platelet activity leads to increased platelet volume.[36] Moreover, MPV correlated positively with WC in all participants in the present study, suggesting that higher abdominal visceral fat is associated with higher MPV concentrations.[37] In the present study, the women had higher PLTs than the men. The mechanisms of sex-related differences in the PLT remain unknown. However, women begin to have a higher PLT than men only after around 14 years of age, supporting the hypothesis that puberty exerts an effect.[38] Our findings suggest that separate reference intervals for men and women are required for analyzing their PLT. The present study had some limitations. We evaluated samples of unequal size in the subgroup analysis, such as the ethnic groups. Further research can be conducted to examine the wider population of diverse ethnic groups in Malaysia, including indigenous minority groups, such as Orang Asli, through a multicenter study. Furthermore, other influential biomarkers of CV risk were not assessed, thus an association between the examined markers and CVD could not be established. We analyzed participants who were not clinically diagnosed as having metabolic syndrome, therefore the present results cannot be extrapolated to all obese individuals. As this was a cross-sectional study, the results must be cautiously interpreted, and a well-designed,

prospective cohort, multicenter study would provide more reliable findings. Finally, multivariable analysis can be performed in the future to account for personal behaviours such as diet, exercise, and substance abuse, in addition to other metabolic variables.

Conclusion

On the basis of the markers used, this study suggests an underlying pathophysiology involving an inflammatory process and possibly increased platelet activation. Although this study suggests that obesity is accompanied by platelet activation (based on the assessment of sP-sel and the MPV), the association of increased PLT with platelet activation could not be established in the obese participants, thus further research is required to investigate this association. However, P-selectin upregulates the tissue factor in monocytes and leads to leukocyte accumulation, which promotes fibrin deposition in areas of vascular injury associated with thrombosis and inflammation.[39] Therefore, sP-sel may be one of several factors contributing to CV risk in overweight and obese individuals. Higher serum sCD40L and sP-sel levels in the women than in the men suggests that obese women are more susceptible to CV risks associated with platelet activation and inflammation. However, this study is the first to evaluate the association of gender with sCD40L and sP-sel levels in obesity. Therefore, these findings must be replicated and cautiously interpreted in a larger sample, considering the limited sample size in our gender-stratified analysis.

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AUTHOR DISCLOSURES

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