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A PRELIMINARY STUDY ON MONOCYTE-PLATELET INTERACTIONS IN DIABETIC SUBJECTS LEADING TO PHENOTYPIC CHANGES IN MONOCYTES'

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ABSTRACT **Background and objectives:** Platelets become hyperactive during progression of inflammatory disease conditions such as diabetes mellitus. We aimed to assess whether activated platelets (CD62+) interact with monocytes to form monocyte-platelet aggregates and such aggregates change the phenotype of monocytes into proinflammatory phenotype (CD14-CD16+) via a comparative study between diabetic and non diabetic subjects. **Methods:** Based on inclusion and exclusion criteria, study subjects were chosen. Monocytes isolated from buffy coat were characterised using immunostaining and flow cytometry. Phenotypic changes in monocytes were determined through flow cytometry; interaction between monocytes and platelets were determined through flow cytometry, ESEM. Proteomic studies were carried out to determine inflammatory marker expression. **Results:** Study revealed that there are monocyte-platelet interactions and alteration in monocyte phenotypes occurring in diabetic subjects compared to control subjects. Western blotting analysis showed the expression of inflammatory molecules IL-10 and MCP-1 in diabetic subjects, further confirming the proinflammatory nature of monocytes. **Conclusion:** Platelet monocytes interaction in diabetic subjects may lead to the phenotypic alteration in monocytes. Inflammatory protein analysis and cell surface marker analysis of monocytes may be considered as a preliminary tool for assessing complications during diabetes mellitus.

KEYWORDS : Monocyte-platelet interaction, Diabetes, Inflammation.

INTRODUCTION

Diabetes mellitus has always been a serious life threatening disorder accompanied with various inflammatory changes involving different cell types such as endothelial cells, platelets, monocytes etc. Platelet activation is a key feature in Diabetes mellitus. Increased glycation products formed via various signalling pathways interact with the membrane proteins present on the surface of platelets making them more prone to activation and interaction with cells [1,2]. High blood glucose levels induce the platelets to express more of their membrane glycoproteins which serve as receptors to interact with other cell types thus increasing their adhesiveness and interaction with them[2]. When platelets get activated they tend to release the proteins stored in their granules which include chemokines that play an important role in interacting and attracting other cell types such as leukocytes to their surface [3]. This interaction serves as a driving factor for formation of platelet aggregates with other cell types. Studies have reported that during inflammation, activated platelets have the tendency to interact with monocytes resulting in the formation of monocyte-platelet aggregates (MPA). MPA are more prone to release inflammatory cytokines into the circulation, the levels of which can be assessed to determine the extremity of the disease progression [4]. Among various cell types, monocytes are one of the most important cell types that form complexes with platelets. Endothelial cells upon encounter with injurious stimuli recruit monocytes to the site of injury. Platelets traps monocytes forming MPA facilitating adherence of monocytes on the surface of endothelial cells. Following this, monocytes migrate into the sub-endothelial space, differentiates into macrophages which imbibe lipid molecules forming foam cells resulting in the development of atherosclerotic plaques[5]. The interaction of platelets with monocytes can cause changes in the latter functionally or phenotypically. Based on the physiological condition to which monocytes are exposed, these cells express specific cell surface markers. Two most common and abundantly expressed are the CD14 and CD16. The percentage of expression of these markers defines the pathological condition of the biological system.

In the current study, focus has been on the comparison of phenotypic changes in monocytes isolated from diabetic and control (healthy) subjects. The objectives of the study were (i) to determine the monocyte-platelet interactions to determine phenotypic alterations in monocytes using flow cytometry; (ii) to determine the expression of inflammatory proteins in monocytes.

MATERIALS AND METHODS

Study subjects and sample collection

Blood samples from control and diabetic subjects were collected from BMT Wing campus SCTIMST, Poojapura as per ethical committee approval approved by Institutional ethical committee (IEC) of SCTIMST, Trivandrum, Kerala, India. (SCT/IEC/818-2015) after taking informed consent from the subjects. All procedures were carried out according to the regulations and guidelines relevant to the research. Diabetic patients (male) belonging to the age group 35- 60 were considered for the study. Diabetes was confirmed as per the International Diabetic Federation (IDF) recommendation of HbA1c and random blood sugar (RBS). HbA1c > 6.5% and RBS > 140 mg/dL were considered as diabetic. Individuals having RBS less than 140mg/dL and HbA1c less than 6.5 were considered as Control [6]. Female population and patients taking anticoagulant drugs were excluded from the study.

Isolation of platelets

Blood was centrifuged at 600g for 5 minutes. Platelet rich plasma was collected centrifuged at 400g for 8 minutes. The obtained pellets were washed with acid citrate dextrose (ACD) :tyroid buffer thrice at 400g for 5 minutes each to get the platelets.

Isolation of monocytes

To optimize monocyte yield two methods for the monocytes isolation were tried. First was monocyte isolation from buffy coat using histopaque density gradient (H) and second was using histopaque-percoll (HP) density gradient [7]. In first method, blood was centrifuged at 600g for 10 minutes. Buffy coat layer formed at the interface between red blood cells and plasma was taken carefully, diluted with phosphate buffered saline (PBS) in the ratio 1:4. Diluted mixture was gently layered on top of 4ml histopaque, centrifuged at 400g for 30 minutes. White layer of cells formed is pipetted out, diluted with PBS (1:2), centrifuged at 400g for three times 6 minutes each to get the pellet and purity of cells was done using flow cytometry. In second method, buffy coat isolated from blood was mixed with PBS in the ratio 1:4, layered on 4ml histopaque, centrifuged at 400g for 30 minutes. White layer formed was collected, diluted with PBS (1:1) and was layered on isosmotic percoll density gradient and centrifuged at 400g for 30 minutes.

Immunostaining

Monocytes isolated using both methods were characterized by

immunostaining. Isolated cells were washed with PBS and fixed in 3.7%formaldehyde for 20 minutes. Cells were blocked with 0.5% bovine serum albumin (BSA) solution. Pellets were washed at 400g for 5 minutes, followed by incubation with CD14-FITC antibody for 1 hour in dark at room temperature. Cells were washed twice with PBS at 400g for 5 minutes each. Cells were observed under inverted fluorescent microscope Olympus lX71 using blue filter.

Flow cytometry analysis

Flow cytometry analysis was carried out (1) to characterise monocytes isolated from buffy coat by histopaque-percoll density gradient method using CD14 antibody; (2) to determine activation status of platelets using CD62-PE ; (3) for determining phenotypic alterations in monocytes using CD14, CD16 antibodies; and (4) to determine monocyte-platelet interactions using CD14, CD62 antibodies. In brief, cells were fixed using 3.7% formaldehyde solution for 20 minutes, followed by wash with PBS at 400g thrice for 5 minutes each. Blocking was done using using 0.5% Bovine Serum Albumin (BSA) solution for 30 minutes followed by PBS washing twice at 400g for 5 minutes each. $1X10^{(5)}$ cells/ml was incubated with primary antibodies (CD14/CD16) for 1 hour at room temperature. Cells were washed at 400g for 5 minutes and were incubated with secondary antibodies tagged with Texas red and Alexa fluor corresponding to CD14 and CD16 respectively for 1 hour in dark at room temperature. Secondary antibodies were used as isotypic control. Cells were washed at 400g for 5 minutes. Cells were analyzed using BD FACS Aria, and flowjo version 7.5 software. 10,000 events were acquired. Compensation was done using single stained cells. Gating was done using unstained population. Experiment was done three times and percentages of cells positive for different CD markers were calculated in control and diabetic subjects. Same procedure was repeated for determining CD62 positive cells which was carried out using PE- tagged antibody.

Environmental scanning electron microscope (ESEM) Analysis

Monocytes and platelets from both control and diabetic subjects were isolated, fixed in 3.7% formaldehyde for 20 minutes. Cells were washed with PBS at 400g for three times five minutes each. Cells were passed through ascending series of alcohol for dehydration. 30%-30 minutes, 50%-30 minutes, 70%-30 minutes, 90%-30 minutes and 100%-30 minutes. Cells suspended in 100% alcohol used for ESEM analysis. The ESEM analysis was carried out using 30Kv Environmentally Scanning Electron Microscope (ESEM- Quanta 200, Germany). Images were taken at magnifications 6000X and 12000X.

Inflammatory proteome analysis

Monocyte proteins were isolated and concentration was determined by Lowry's method. SDS PAGE was done for monocyte proteome using 12% resolving gel and 5% stacking gel and stained with Coomassie brilliant blue R- 250. Gels were documented using Alpha Imager Documentation System 2000. Western blotting was performed in order to determine the expression of inflammatory molecules using polyvinylidene fluoride (PVDF) membrane (25V, 0.1A, 1hour) at room temperature. Membrane was washed in PBS with tween 20 (PBST) buffer for 2-3 times 5 minutes each and blocked using 3% BSA for 1 hour, washed in PBST buffer and incubated with primary antibodies interleukin-10 (IL-10) and monocyte chemoattractant protein (MCP- 1) at 4°C overnight. Two different dilutions of antibodies were used, 1:500 and 1:1000. Following incubation, the membrane was washed with PBST thrice and incubated with secondary antibody for 2 hours at room temperature with continuous shaking. After 2 hours the membrane was washed with PBST and allowed to develop using the 3,3'-diaminobenzidine (DAB) developer solution in dark. Once the specific band was developed the development was stopped using distilled water.

Statistical analysis

Statistical analysis was carried out for all the quantitative data. Student's t-test was used to determine significant difference based on p-value. Values are represented as Mean±SD. P<0.05 were considered to be significant.

RESULTS

Screening of subjects

Subjects were screened based on RBS and HbA1c level. Subjects who qualified the IDF criteria were recruited in the study. Fig 1(a) indicates blood glucose levels (80.2±7.39 in control subjects; 266.6±87.72 in diabetic subjects) and (b) HbA1c levels (5.22±3.0 in control subjects and 7.13±1.0 in diabetic subjects).

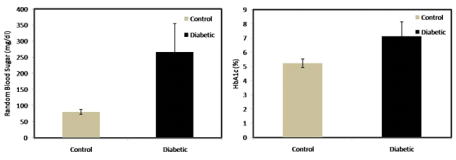

Figure 1: Comparison of blood glucose levels (a) HbA1c levels (b) in control and diabetic patients. Values are represented in Mean±SD (n=5). P<0.05.

Activation status of platelets by flow cytometry

Platelets from control and diabetic subjects were analyzed by flow cytometry to detect the expression of surface marker CD62. X-axis represents the flurochrome and Y-axis represents the count. The representative data from one patient has been shown in Fig II. Results indicate that CD62 (P-selectin) expression increases in diabetic subjects compared to control. The red line represents the basal level expression of CD62 in control subjects. The blue line represents the expression of CD62 in diabetic subjects. So it is clear that compared to control subjects, diabetic subjects showed approximate 15% positivity hike for CD62.

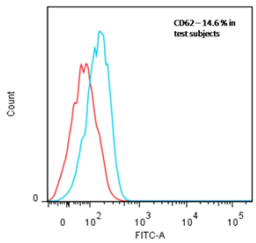

Figure 2: FACS analysis for platelet activation in control and test subjects. Red line denotes the control subjects with basal level expression of CD62 whereas blue denotes the expression of CD62 in diabetic subjects (n=5).

Characterisation of monocytes by Immunostaining and Flow cytometry

Monocytes isolated from the buffy coat by two methods were characterised for their purity and was analyzed by immunostainning and flow cytometry. So, HP method was performed to improve the yield of monocytes. Fig 3-(i) (a) and (b) represents the CD14 stained monocytes isolated from blood using histopaque. (c) and (d) represents CD14 stained monocytes isolated from blood using double density gradient (histopaque followed by percoll gradient). Cells were stained with CD14-FITC and images were captured at 20X magnification using inverted fluorescent microscope Olympus lX71. Further the CD14 positive cells were determined quantitatively by assessing the intensity using Image J software and the results obtained is graphically represented in fig 3-(ii) which shows that CD14 positive cells are found to be higher when isolated using HP method compared to H method with significant difference among H and HP methods in case of diabetic subjects. Thus for the further analysis double density gradient method was used for monocyte isolation. Purity of isolated monocytes by double density gradient method was further quantified by flow cytometry using CD14 antibody. Representing FACS data is shown in fig 4. The red line shows the basal level expression of CD14 in unstained population and blue line represents the CD14 positive cells in control subjects. Here the graph represents fluorochrome on X-axis and count on Y-axis. X-axis represents the % of fluorochrome positive cells. More than 85% cells were CD14 positive in control subjects isolated by HP method suggesting it as a better method for monocyte isolation. This experiment was carried out in order to determine which method of monocyte isolation yields better positivity. Once standardized, by flow cytometry and immunostaining, monocyte isolation for further experiments from control and diabetic subjects were carried out using double density HP method.

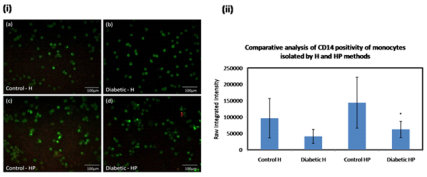

Figure 3: (i) CD14-FITC immunostained images of cells isolated from

control (a) and test subjects (b) by histopaque (H) method; Histopaque Percoll (HP) method control (c) and (d) test subjects. Images were captured at 20X magnification. (ii) Graphical representation of quantitative analysis of CD14 positive monocytes isolated from control and diabetic subjects using Histopaque (H) and Histopaque-Percoll method (HP). *P<0.05. significance in CD14 positivity in cells isolated from diabetic subjects using HP method is represented with respect to cells isolated from diabetic subjects using H method.

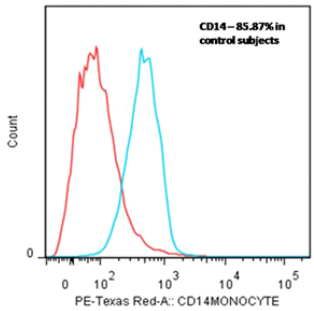

Figure 4: FACS analysis of CD14 positive monocytes. Red line shows the basal level expression of CD14 in unstained population and blue line represents the CD14 positive cells in control subjects.

Platelet monocyte interaction analysis by ESEM

Images of MPA formed captured at different magnifications are represented in fig 5. Interactions can be seen in the form of MPA. Smaller cells with extended pseudopodia like structures are the platelets which can be seen adhered to the surface of monocytes. Larger cells are the monocytes which are typical round in shape. It is evident from the fig 5, that in diabetic subjects fig 5 (c) and (d), the number of MPA formed are extremely higher compared to the control subjects figure 5 (a) and (b). Platelet - platelet interactions are found to be less in control subjects compared to diabetic subjects. Thus, ESEM images confirm that there is an interaction occurring between monocytes and platelets which stimulate them to form MPA.

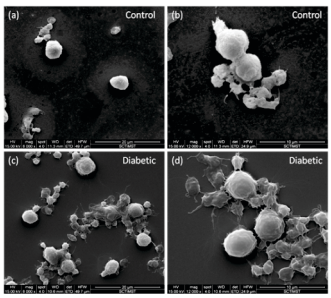

Figure 5: ESEM images showing platelet-platelet (P-P) and monocyte-platelet (M-P) interactions in control (a), (b) and diabetic individuals (c), (d). Images were captured at 6000X and 12000X magnifications.

Platelet monocyte interaction by flow cytometry

Flow cytometry analysis was done to determine monocyte platelet interactions occurring in control and diabetic subjects. The fig 6 shows the percentage of monocyte platelet interactions in control and diabetic subjects. MPA formation was quantified using flow cytometry. Here the individual graph represents CD14 on X- axis and CD62 on Y-axis. Fig 6 represents the FACS data, where dual positive cells (CD14+/CD62+) were considered as aggregates and extent of formation of heteroaggregates is well correlated with the platelet activation. Heteroaggregates population was found to be 7.93±6.2 in control and 9.89±5.3% in diabetic subjects (Fig 6 d).

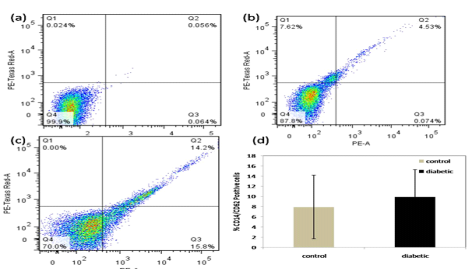

Figure 6: FACS analysis of monocyte platelet interactions. (a) unstained population of monocytes and platelets (b) stained monocyte platelet population from control subjects. (c) stained population of monocytes and platelets from diabetic subjects. (d) Graphical representation of FACS data. Values are represented in Mean±SD (n=3).

Phenotypic variation in monocytes

For analyzing phenotypic alterations in monocytes, flow cytometry of monocytes in control and diabetic subjects were performed. The fig 7 shows the percentages of phenotypic alterations in the monocytes isolated from control and diabetic subjects. Here the individual graph represents fluorochrome on X-axis (CD14) and side scatter on Y-axis (Cd16). It was observed that CD14/CD16 positive population was high (12.21±3.65%) in diabetic subjects as shown in fig 7(c) compared to the healthy individuals (9.44±3.22%) as shown in fig 8 (b). Fig 7 (d) represents the comparative graphical representation of CD14/CD16 positive population between control and diabetic subjects. A marginal increase was observed in dual positive cells in diabetic subjects compared to control subjects.

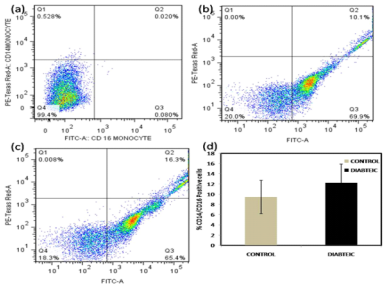

Figure 7: FACS analysis of phenotypic alteration in monocytes. (a) represents unstained monocytes. (b) represents CD14/CD16 stained monocytes from control subjects. (c) represents CD14/CD16 stained monocyte population from diabetic subjects. (d) graphical representation of comparison of degree of phenotypic alterations in control and diabetic subjects. Values are represented in Mean±SD (n=3).

Analysis of inflammatory markers

High concentration of protein was observed in the diabetic subjects compared to the healthy individuals (fig 8 (a)), which indicates that synthesis or the secretion of proteins might increase upon activation of the monocytes. Representative SDS-PAGE image is shown in fig 8 (b) with the molecular weight marker (10-250kDA). SDS images also show more number of bands in the test group compared to the control. Western blot analysis showed increase in the levels of both inflammatory proteins, which indicates that monocytes in diabetic subjects are in inflammatory stage and releases inflammatory marker proteins.

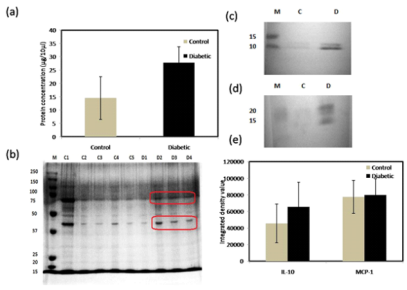

Figure 8: (a) Comparison of protein concentrations in control and diabetic subjects. Values are represented in Mean±SD. P-value<0.05. (b) SDS PAGE images showing different protein bands of monocyte proteome isolated from control and diabetic subjects. Molecular weight range of protein marker (10-250kDa). C1-C5 represents monocyte proteins from control subjects. D1-D4 shows monocyte proteins from test subjects. (c) Western blot image for MCP-1:11-13kDa. M-Protein marker (molecular weight range: 10kDa -250kDa), C-Control sample, D-Diabetic sample. (d) Western blot image for IL-10: 18-20kDa. M-Protein marker (molecular weight range: 10kDa - 250kDa), C- Control sample, D-Diabetic sample. (e) Graphical representation of band intensity of respective inflammatory markers quantified by alpha imager gel doc-system.

DISCUSSION

Inflammatory disorders are often associated with activation of cells of biological system as a result of effect from several inflammatory mediators. Diabetes is one such disorder accompanied with chronic inflammatory changes and is regarded as one of the most life threatening disease condition with several complications such as CVDs which is accompanied with hypertension, hypercholestremia and other associated vascular complications [8,9]. In this study study subjects were selected based on two criteria that is (i) random blood sugar and (ii) HbA1c levels in blood plasma. HbA1c is glycated haemoglobin which is formed when the blood haemoglobin gets glycated by the glucose present in the blood plasma. HbA1c gives the average level of glucose in plasma over a prolonged period of time. If a subject is having continued high levels of glucose in the blood it will be reflected in the HbA1c concentrations[10].

Platelets and monocytes from both control and study subjects were isolated. As described in the methodology section, two methods were tried for monocyte isolation. And it was observed that cells isolated via double density gradient showed higher CD14 positivity. Thus for further analysis cells were isolated using double density gradient method. Flow cytometric analysis of isolated cells showed around 85% CD14 positive cells. CD14 has been considered as a specific marker for monocyte identification and such monocytes are considered to be phagocytic and non-inflammatory in nature[11].

Platelets play an important in maintaining haemostasis. Soluble factors like ADP, thrombin released by activated cells promote the intracellular calcium levels in platelets activating signalling pathways leading to platelet activation[12,13]. P-selectin is a membrane marker which gets exposed even on mild activation of platelets. It is of gold standard marker considered for platelet activation studies[14]. In diabetic subjects as well as in high risk groups like hypertension/ hypercholesterolemia, platelets are reported to be in their activated stage[15]. P-selectin mediates rolling of monocytes on activated endothelium. In our study platelets showed activated status of around 14.62% positivity for CD62. In activated platelets, P- selectin is translocated from the alpha granules to surface of plasma membrane and mediates adhesion of platelet to neutrophils and monocytes. It has been reported that in severe inflammatory infectious diseases these activated platelets play an important role in forming aggregates with leukocytes especially the monocytes [16]. It is clear from the data obtained that diabetic subjects have activated platelets in the circulation which may interact with monocytes to form monocytes platelet aggregates (MPA). These MPA is clearly visible in ESEM images and have been quantitatively analyzed via flow cytometry. It is evident from the images that platelets spread through the pseudopodia and form aggregates with monocytes in diabetic group while being not prominent absent in the control group. Thus it is suggested that activated platelets has got high affinity towards monocytes, forming monocyte platelet aggregates with the help of various cell adhesion molecules that are expressed on the surface of both cells [17]. Detection of MPA in patients can serve as a method for early detection of proinflammatory conditions suggesting its use as a pathophysiological marker in chronic inflammatory disorders. Increased presence of MPA serve as an early detection marker of type 2 diabetes [17]. It also depicts increased risk of atherosclerosis [17]. MPA have also been found in acute coronary syndromes [16]. MPA thus help in attachment of monocytes to endothelial cells of arterial blood vessels and contribute to atherosclerotic plaque formation thereby contributing to enhanced endothelial dysfunction[18]. Higher the platelet activation, higher the percentage of MPA formed. Activated platelets and their interactions with other cell types may leads to alteration of the monocytes phenotype to the proinflammatory CD14+/CD16++ phenotype, which may in-turn release the inflammatory cytokines and proteins which are primary responsible for the progression of inflammatory diseases and susceptibility of diabetes to the cardiac diseases. The percentage of non-classical monocytes have been found to be increased during chronic inflammatory disorders[11]. There are three subsets of monocytes reported CD14++/CD16- the classical subset, CD14+/CD16++ the non-classical and CD14++/CD16+ the intermediate population. Monocytes are found as classical subset of CD14++/CD16- in normal physiological conditions; in pathological conditions intermediate subset CD14++/CD16+ and proinflammatory subset CD14+/CD16++ increases, however not much is being reported about the presence of these subsets in diabetic subjects and their role in the inflammation in diabetes. Major difference in subsets are the expression of specific cell surface markers that are expressed on CD16 exclusively such as MDC8, DR, EMR2, ILT-4, CD45Ra etc which are not expressed or slightly expressed by CD14+ cells thus making CD16 more pro- inflammatory in nature [19]. Our study demonstrated higher levels of CD14+/CD16++ subsets in diabetic subjects. It has also been reported that CD16 expression increases in cardiovascular diseases which is one of the major complications developed during chronic diabetic conditions[20]. The interactions observed in our study may in turn cause release of inflammatory proteins by monocytes. Inflammation plays an important role in phenotypic alteration of monocytes and there occurs a shift from classical monocyte population to non- classical monocyte population[21]. In our study we explored the inflammatory markers secreted by monocytes to understand the impact of monocytes platelet interaction on inflammation. Proteomic analysis showed higher concentration of total protein in diabetic subjects compared to control subjects. It might be due to the fact that in diabetic patients who are often associated with cardiovascular complications are found to have increased serum protein concentrations. The most abundant plasma protein in human is albumin which is increased in inflammatory conditions [22]. As a result of inflammation, several inflammatory/anti-inflammatory cytokines are released by monocytes. Two most important one are the IL-10 and MCP-1. IL-10 also known as cytokine synthesis inhibitory factor (CSIF) is an anti-inflammatory molecule. It suppresses the secretion of other cytokines in order to reduce inflammation. During type 2 diabetes, monocytes produce IL-10 so that other inflammatory cytokines are reduced in circulation [23]. MCP-1 is an inflammatory cytokine secreted by monocytes. It is a chemokine that acts as an important chemoattractant for the migration of monocytes and macrophages to the inflammatory site[23]. Upon analysis of inflammatory markers via western blotting, revealed the presence of both IL-10 and MCP-1 in higher intensity in diabetic subjects compared to control subjects. Thus monocytes in diabetic subjects are proinflammatory with slight phenotypic alteration in non classical CD16+ population. In conclusion, we have attempted to understand the effect of platelet monocyte interaction on non classical monocyte population. A mild increase in the nonclassical population was observed in diabetic subjects, which could be the reason for high inflammatory protein expression. This study might help clinically to assess the intensity with which diabetic patients are prone to CVDs based on the quantification of monocyte-platelet aggregates formed. This is the first time study that has correlated the quantification data of monocyte-patelet aggregates with visual observations of the same by ESEM. The study projects a combinatorial approach of determining the interaction between both cell types qualitatively and quantitatively. Though large population study may be helpful, this study gives an insight about the phenotypic changes in monocytes in diabetic subjects. However, the major limitation of the study was the low number of patients selected for the study which was due to the lack of availability of patients fitting to our selection criteria was very few.

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Conflict of interest
Nil

REFERENCES

1. Ferroni P, Basili S, Falco A, Davì G. Platelet activation in type 2 diabetes mellitus. Journal of Thrombosis and Haemostasis. 2004;2(8):1282–91.
2. Schneider DJ. Factors Contributing to Increased Platelet Reactivity in People With Diabetes. Diabetes Care. 2009 Apr;32(4):525–7.
3. Randriamboavonjy V. Mechanisms Involved in Diabetes-Associated Platelet Hyperactivation. The Non-Thrombotic Role of Platelets in Health and Disease [Internet]. 2015 Nov 18 [cited 2019 Feb 11]; Available from: https://www.intechopen.com/books/the-non-thrombotic-role-of-platelets-in-health-and-disease/mechanisms-involved-in-diabetes-associated-platelet-hyperactivation
4. Major HD, Cambell RA, Silver RM, Branch DW, Weyrich AS. Synthesis of sFlt-1 by platelet-monocyte aggregates contributes to the pathogenesis of preeclampsia. Am J Obstet Gynecol. 2014 Jun;210(6):547.e1-547.e7.
5. Meng L, Park J, Cai Q, Lanting L, Reddy MA, Natarajan R. Diabetic conditions promote binding of monocytes to vascular smooth muscle cells and their subsequent differentiation. Am J Physiol Heart Circ Physiol. 2010 Mar;298(3):H736–45.
6. Ceriello A, Colagiuri S. International Diabetes Federation guideline for management of postmeal glucose: a review of recommendations. Diabet Med. 2008 Oct;25(10):1151–6.
7. Almeida MC de, Silva AC, Barral A, Barral Netto M. A simple method for human peripheral blood monocyte Isolation. Memórias do Instituto Oswaldo Cruz. 2000 Apr;95(2):221–3.
8. Tracey ML, McHugh SM, Fitzgerald AP, Buckley CM, Canavan RJ, Kearney PM. Risk Factors for Macro- and Microvascular Complications among Older Adults with Diagnosed Type 2 Diabetes: Findings from The Irish Longitudinal Study on Ageing [Internet]. Journal of Diabetes Research. 2016 [cited 2019 Mar 31]. Available from: https://www.hindawi.com/journals/jdr/2016/5975903/
9. Couppé C, Svensson RB, Grosset J-F, Kovanen V, Nielsen RH, Olsen MR, et al. Life-long endurance running is associated with reduced glycation and mechanical stress in connective tissue. Age (Dordr) [Internet]. 2014 Aug [cited 2019 Feb 17];36(4).

Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4150896/
10. Huang W, Vodovotz Y, Kusturiss MB, Barclay D, Greenwald K, Boninger ML, et al. Identification of Distinct Monocyte Phenotypes and Correlation with Circulating Cytokine Profiles in Acute Response to Spinal Cord Injury: A Pilot Study. PM & R : the journal of injury, function, and rehabilitation. 2014 Apr;6(4):332.
11. Yun S-H, Sim E-H, Goh R-Y, Park J-I, Han J-Y. Platelet Activation: The Mechanisms and Potential Biomarkers. BioMed Research International [Internet]. 2016 [cited 2019 Feb 18];2016. Available from: https:// www. ncbi. nlm. nih. gov/pmc/articles/ PMC4925965/
12. Herter JM, Rossaint J, Zarbock A. Platelets in inflammation and immunity. Journal of Thrombosis and Haemostasis. 2014;12(11):1764–75.
13. Ju L, McFadyen JD, Al-Daher S, Alwis I, Chen Y, Tønnesen LL, et al. Compression force sensing regulates integrin α IIb β 3 adhesive function on diabetic platelets. Nature Communications. 2018 Mar 14;9(1):1087.
14. Rondina MT, Carlisle M, Fraughton T, Brown SM, Miller RR, Harris ES, et al. Platelet–Monocyte Aggregate Formation and Mortality Risk in Older Patients With Severe Sepsis and Septic Shock. J Gerontol A Biol Sci Med Sci. 2015 Feb;70(2):225–31.
15. Mezzapesa A, Bastelica D, Crescence L, Poggi M, Grino M, Peiretti F, et al. Increased levels of the megakaryocyte and platelet expressed cysteine proteases stefin A and cystatin A prevent thrombosis. Scientific Reports. 2019 Jul 3;9(1):9631.
16. Patkó Z, Császár A, Acsády G, Öry I, Takács É, Fűrész J. Elevation of monocyte–platelet aggregates is an early marker of type 2 diabetes. Interv Med Appl Sci. 2012 Dec;4(4):181–5.
17. Passacquale G, Vamadevan P, Pereira L, Hamid C, Corrigall V, Ferro A. Monocyte-platelet interaction induces a pro-inflammatory phenotype in circulating monocytes. PLoS ONE. 2011;6(10):e25595.
18. Ziegler‐Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. Journal of Leukocyte Biology. 2007;81(3):584–92.
19. Mandl M, Schmitz S, Weber C, Hristov M. Characterization of the CD14++CD16+ Monocyte Population in Human Bone Marrow. PLOS ONE. 2014 Nov 4;9(11):e112140.
20. Liang H, Duan Z, Li D, Li D, Wang Z, Ren L, et al. Higher levels of circulating monocyte–platelet aggregates are correlated with viremia and increased sCD163 levels in HIV-1 infection. Cell Mol Immunol. 2015 Jul;12(4):435–43.
21. Riaz S, Tariq M, Aslam S. Association of Serum Protein Levels in the Diabetic Patients with Risk of Cardiovascular Disease and Nephropathy in Pakistani Population. Journal of Research in Diabetes. 4(1):5.
22. Yaghini N, Mahmoodi M, Asadikaram GR, Hassanshahi GH, Khoramdelazad H, Kazemi Arababadi M. Serum Levels of Interleukin 10 (IL-10) in Patients with Type 2 Diabetes. Iran Red Crescent Med J. 2011 Oct;13(10):752.
23. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. J Interferon Cytokine Res. 2009 Jun;29(6):313–26.