Expression of Tie2 (angiopoietin receptor) on the monocyte subpopulations from ischemic stroke patients: Histological and flowcytometric studies

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Article

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Abstract

Introduction: Different monocytes subpopulations have a distinct role as phagocytic subsets but also act as pro-inflammatory and pro-angiogenic subsets (i.e.Tie2-expressing monocytes, or TEMs).Recruitment of the monocyte-derived macrophages to the affected brain occurs most abundantly between 3–7 days after the occurrence of ischemic stroke. Aim:To investigate the expression levels of Tie2 (a receptor of angiopoietin) on monocytes and their subpopulations using histological and immune-histological study of bone marrow biopsies and blood flowcytometric examination from ischemic stroke patients.

Methods:Ischemic stroke patients were selected within 2 days after clinical confirmation by medical consultants. Healthy volunteers matched with age and sex participated as the control group. Bone marrow trephine biopsies from the iliac crest were performed and fixed for histological and immunohistological staining by anti CD14 and CD68. Fresh blood samples were collected and analyzed by flowcytometry for total monocytes, monocyte subpopulations and Tie2 expressing monocytes (TEMs), following staining with CD45, CD14, CD16, and Tie2 monoclonal antibodies.

Results:Examination of poststroke patients' bone marrow cells demonstrated hypercellularity. There were an apparent increase of CD 68 and CD 14 positive cells expression. Also, the ischemic stroke patients exhibited a significant increase in the percentage of intermediate monocytes $CD14^{high}CD16+$ with a low percentage of non-classical $CD14^{low}CD16^{++}$ monocytes. In addition,Tie2 expressing monocytes were significantly higher in ischemic stroke patients compared to the control group.

Conclusion: In conclusion, the current results demonstrated dysregulation of the angiogenic function of monocytes subsets, which could be an early diagnostic marker to start either a neurovascular repair therapy or improve medications attributed to highly senescent subpopulations.

Introduction

Stroke is a highly destructive illness worldwide it is a third major contributor to disability and mortality. According to W.H.O about 16.9 million people in the world suffer from the stroke in every year. Majority of the stroke cases happen due to ischemic stroke while other cases occur because of hemorrhagic. It is important to note that if the trend in stroke mortality, disability, and prevalence continue, about 12 million of the stroke deaths with more than 200 million of disability cases will occur worldwide by the year 2030. It is reported that about 80 percent of the stroke cases worldwide happen due to ischemia (1).

In Saudi Arabia, according to statistics, cardiovascular disease represents 22 % of death (2). Research carried out in Saudi Arabia revealed that the estimated rate of stroke cases is 29.8 per of 100,000 cases (3). With increasing risk of stroke, especially with the increase of diabetes mellitus (DM) as one of the major risk factors for the disease. DM poses about four times higher risk for stroke (4). In a society like Saudi Arabia, which has had an epidemic of diabetes in the last three decades, stoke is one of the worst complications for DM. Additionally, the high association of disease with both mortality and disability

especially in developing country, there is an urgent demand for invention of new therapeutic approaches for stroke (5).

Because of high association of stroke with both mortality and disability, there is an urgent demand for invention of new therapeutic and diagnostic approaches. The pathophysiology of ischemic stroke remains unclear.Recently, it has been demonstrated that the pathophysiological responses of stroke consist of acute neurovascular injury that damage the blood–brain barrier (BBB) and lead to neural cell death. In addition, delayed neurovascular repair (angiogenesis, neurogenesis) is involved in the stroke pathogenesis (6). Different monocytes subpopulations have a distinct role as phagocytic subsets but also act as pro-inflammatory and pro-angiogenic subsets (i.e., Tie2-expressing monocytes, or TEMs) (7). The angiogenesis and neuro-angiogenesis has been described in the stroke patients. Despite the critical role of Tie2 and monocytes subsets in initiation and progression of angiogenesis, and involvement in the management of stroke patients (8). The expression levels of Tie2 (a receptor of angiopoietin) on monocytes and their subpopulations using histological and immune-histological study of bone marrow biopsies and blood flowcytometric examination from ischemic stroke patients.

Material And Methods

Tissue preparation:

Bone marrow trephine biopsies were done in selected cases. The core specimens were placed in 10% Phosphate Buffered Formalin (PBF). The fixed tissues were processed using automated tissue process of the Neurology lab unit of King Abdelaziz university. The paraffin blocks were cut with a rotary microtome at 3-5 µm thickness. The sections were floated on a warm water bath at 40°C and taken on a glass slide and incubated for 24 hours. The sections were allowed to cool and stained with Hematoxylin and Eosin (H&E) to assess the structural changes under the light microscope.

For immunohistochemical staining, serial paraffin sections were chosen to be stained by 2 markers for detection of the monocytes. The slides were fixed, deparaffinized and hydrated, treated with 0.5% hydrogen peroxide in methanol for 10 min to block endogenous peroxidases, and washed in tap water. Sections were incubated with 10 mM citrate buffer, pH 6.5, and heated in a microwave oven for two cycles of approximately 2 min each for antigen retrieval. After cooling, sections were washed in PBS (pH 7.6) for 5 min. and placed in normal saline. Sections were incubated with anti CD14 mouse primary antibodies for 2 h from (Novocastra, France). The binding of anti-CD14 mouse antibody was revealed by biotinylated goat anti-rabbit and anti-mouse antibody (AbCys, Paris, France), followed by streptavidin–peroxidase complex (AbCys, Paris, France). The peroxidase activity was developed by using diaminobenzidine (DakoCytomation, Trappes, France). Other selected sections were incubated with anti-CD68 (PG-M1) (DAKO, Denmark) mouse antibody for 1 h. The binding of anti-CD68 antibody was revealed by rabbit anti-mouse antibody, followed by incubation with 1 mg/ml DAB solution (3,3tetrahydrochloride diaminobenzidine, Sigma, USA) for 5 min. Anti CD68 is specific for staining the

macrophages developed from the monocytes. The slides were counterstained with Mayer's hematoxylin and mounted in DPX. Then the sections were completely scanned, examined and photographed.

Blood samples:

Immunophenotyping studies were done on the fresh blood samples of patients with ischemic stroke after diagnosis was confirmed by medical consultants. The peripheral whole blood samples (5 ml) is collected in Ethylenediaminetetraacetic acid (EDTA) tubes. (Becton Dickinson, San Jose, USA), ethical approval No 53217. Full blood counts were performed using a Sysmex XS whole blood analyzer (Sysmex Corporation, Kobe, Japan). Immunophenotyping studies were performed on a total of 12 newly diagnosed with ischemic stroke patients from 2020 (King abduaziz university hospital).

Immunophenotyping

Fluorochrome-conjugated CD markers are purchased from Bio Legend, USA. A combination of peridinin chlorophyll protein (PerCP), fluorescein isothiocyanate (FITC), allophycocyanin (APC), and phycoerythrin (PE) conjugated antibodies were selected. CD16- FITC (monocytes), CD14- PerCP/Cy5.5 (monocytes), CD202b (Tie2) and CD45-APC (leukocyte) were used for leukocyte immunophenotyping (surface staining) using the FACS Canto™ II (Table 1).

Table 1: List of antibodies used for flowcytometry

Materials	Catalogue number	Supplier
PE anti-human CD202b (Tie2) Antibody	334206	BioLegend (San Diego, USA)
PerCP/Cy5.5 anti-human CD14 Antihody	367110	BioLegend (San Diego, USA)
FITC anti-human CD16 Antibody	360716	BioLegend (San Diego, USA)
APC anti-human CD45 Antibody	20021658	Dako (CA, USA)

Flowcytometer Calibration

BD FACS Diva CS&T IVD Beads

BD FACS Diva CS&T beads were used to set up the cytometer, to perform daily performance quality control (QC), and to determine lyse/wash (LW) application settings, allow the software to automatically characterize, track, and report measurements of the cytometer. Automated algorithms in the software defined the cytometer baseline. Once baseline mean fluorescence intensity (MFI) target values were defined, the beads were used to run daily performance checks. BD FACS Diva CS&T IVD beads are also used to reset MFI target values when switching to a new lot of beads. In addition, BD FACS Diva CS&T

IVD beads were used to manually determine LW application setting LW once saved, application settings were automatically cytometer after the user performs the dieter performance check the instrument performance, base once that day.

Fluorescence compensation

Using BD™ Comp Beads Set Anti-Mouse Ig, κ are polystyrene microparticles which are used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. The set provides two populations of microparticles, the BD™ Comp Beads Anti-Mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, and the BD™ Comp Beads Negative Control, which has no binding capacity. Four tubes each containing 100 µl of staining buffer BSA , were stained with CD16 -FITC, CD202-PE, CD14-PerCP and CD45-APC (positive control) and one tube containing 1 x 106 white blood cells were subjected to sample processing, with equivalent volumes of PBS in place of the CD markers (unstained cells) for electronic optimization. One drop of BD™ Comp Beads Anti-Mouse Ig, κ was added to positive control tubes and one drop of Comp Beads Negative Control was added to unstained tube (negative control). All incubations were performed at ambient temperature, unless otherwise stated. Cells were stained with CD markers for 15 minutes, in the dark. Unstained tube was then incubated in 2 mL FACS Lyse solution (BD Biosciences, San Jose, U.S.A) for 10 min, in the dark. Cells were then centrifuged at 300 x g for 5 min, washed in 2 mL cell wash, and re-centrifuged at 300 x g for 3 min.

Forward and Side Scatter Optimization

Forward scatter (FSC) amplification (amp) gain and CD45 were adjusted to position the three main leukocyte subpopulations; lymphocytes, monocytes and granulocytes on the linear scale. FSC threshold was adjusted to minimize debris appearing on the scale. FSC and CD45 were optimized for all samples under all conditions. Lymphocyte and monocyte populations were gated using a conjugated-CD45 vs. SSC plot. 1.0 x 106 cells were stained with CD45-PerCP or CD45-PE-CyTM5 to facilitate gating the main types of leukocytes (granulocytes, monocytes and lymphocytes), and to gate out debris. Data acquisition and analysis were completed using the a BD FACS CantoTM II software (CA, USA). The 20,000 events were acquired for each sample.

Expression of surface antigens on monocyte subsets:

The Procedure for analysis of surface antigens used 3 BD Falcon FACS tubes. Tube 1 was the surface negative tube and tube 2 included all antibodies and tube 3 was used as an internal control. Whole blood (100µl) was added to each 3 BD Falcon tubes, then CD 45 was added to all tubes. Next, CD14 and CD16 were added to tube 2 and tube 3. The CD202 was added exclusively to tube 2. All tubes were incubated for 15 minutes in the dark. Then 2ml of BD FACS lysing solution was added to each tube, then incubated for 10 minutes. Then all tubes were washed 2 times with Cell wash PBS. The last step involved adding 500uL to each tube and then it was input using FACS Canto™ II Flow Cytometer.

Statistical Analysis

In this study, Student's T-test was performed to compare the difference in mean SD. GraphPad Prism version 8.00 for Mac Correlation coefficient was made using Person test. Correlation is significant at the p value ≤ 0.05 level (2-tailed). The analysis and graphs were done by, GraphPad Software, La Jolla California USA, www.graphpad.com".

Results

Study population: The demographics data of the included subjects in this study were listed in Table (2). Patients with ischemic stroke were well matched with controls for age and sex.

Table 2: Demographics and clinical criteria of ischemic stroke patients and age-matched controls

	Control (n=20)	**Ischemic stroke patients (ISP)** (n=22)
Age (years)	55 (44-60)	55 (44-65)
Male	6 (60.00%)	7 (58.33%)
Female	4 (40.00%)	5 (41.67%)
Positive smoking history	-	3 (25.00%)
Hypertension	-	6 (50.00%)
Type 2 diabetes mellitus	-	3 (25.00%)

Histology Results:

Examination of H&E sections from the trephine biopsy of the bone marrow from the control subjects revealed the normal structure of the human bone marrow with different clusters of red blood cell (RBCs) series, WBCs series, megakaryocytes in between the blood sinusoids. The acidophilic bone trabeculae appeared radiating inbetween the bone marrow tissue (Fig.1A). A higher magnification of most of examined sections demonstrates the presence of the erythroid series, granulocyte series, and megakaryocytes (Fig. 2A).

Examination of H&E sections from the trephine biopsy of the bone marrow from the Ischemic stroke patient displayed a hypercellular bone marrow between bone trabeculae with apparent decrease of the adipose tissue (Fig.1B).

A higher magnification of most of examined sections displayed an apparent increase in granulocyte series on expense of the erythroid series. The megakaryocytes were easily detected between the bone

marrow cells and dilated blood sinusoids (Fig. 2B).

Examination of CD14 immunostained sections from the control subjects showed the sparce distribution of the brown stained CD14 positive cells of monocytes (Fig.3A). The CD14 immunostained sections of the ischemic stroke patients demonstrated an apparent increase in the clusters of the CD14 brown stained immunopositive cells compared to the examined control sections (Fig. 3B).

Examination of CD68 immunostained sections from the control subjects displayed the sparce number of the brown stained CD68 positive cells (Fig.4A). The CD68 immunostained sections of the ischemic stroke patients showed an apparent increase in the CD68 brown stained immunopositive cells compared to the examined control sections (Fig. 4B).

The flowcytometric results:

The total leukocytes in ischemic stroke patients were significantly higher than the control subjects ($P<0.05$) (Figure 5.A). The total number of monocytes was significantly higher amongst cases versus control group ($P<0.001$) (Figure 5.B).

Monocytes were separated according to their expression of CD14 and CD16 into the three main monocyte subsets: classical CD14highCD16-, non- classical $CD14^{low}CD16^{+}$ and intermediate CD14+CD16+ (Figure 6). By contrast, differences in monocyte subpopulations were prevalent between the three groups. Compared to the control group, ischemic stroke patients exhibited a highly significant greater proportion of intermediate monocytes CD14+CD16+ ($P<0.001$) (Figure 7.A) but exhibited a significant lower proportion of non- classical $CD14^{low}CD16^{+}$ monocytes ($P<0.05$) (Figure 7.B). Meanwhile, classical monocyte with CD14highCD16- showed nonsignificant difference between patients and control ($P<0.489$) (Figure 7.C).

Percentage of TEMs in peripheral blood monocytes in different groups

Tie2 expressing monocytes were significantly higher in ischemic stroke patients compared to the control. Tie2 expression was compared in circulating monocytes from patients with ischemic stroke and matched controls using flow cytometry. The circulating TEMs was significantly higher in ischemic stroke patients compared to control group ($P<0.001$). (Figure 8.A). Furthermore, the expression of Tie2 on Intermediate monocytes (pro-angiogenic phenotype) was highly significant increase in stroke patients compared with the control group ($P< 0.001$) (Figure 8.B). The Tie2 on non-classical monocytes (proinflammatory phenotype) were highly significant increase in ischemic stroke patients compared to the control subjects ($P<0.001$) (Figure 8.C). The Tie2 on Classical Monocytes was highly significant increase in ischemic stroke patients compared to control subjects ($P<0.001$) (Figure 8.D).

By analyzing with the percentage of TEMs in peripheral blood monocytes of stroke patients, there was a significant positive correlation between percentage of Tie2 on classical monocytes and Tie-2 On total monocytes percentage ($r=0.622$; $P<0.05$) (Figure 9).

Discussion

Monocytes are the target in the stroke since they are main cells of innate immunity which can also influence adaptive immunity (9). Monocytes can be differentiated to macrophages and dendritic cells, and they are critical when the immune response to pathogens and a number of endogenous molecules, such as heat-shock-protein, RNA, fibronectin, or fibrin, is initiated (10). In the current study, It is reported that ischemic stroke patient revealed an apparent decrease of adipose cells and hypercellularity with increased vascularity in bone marrow sections stained with Hx&E stain. Similar picture was detected by some investigators (11,12 &13). Yang et al. presumed that mononuclear cells within the bone marrow fraction may change in quantity and quality compared to the mononuclear nuclear bone marrow cells of healthy animals following a brain injury such as stroke. They investigated this problem by comparing mononuclear cells produced before and after a stroke from the same animals (13).

This is a key question, since a recent study showed that the stroke in leukocyte response fluencies is known to regulate the autonomic regulation of bone marrow by the central nervous system (12). Courties et al. stated that myeloid cells, particularly neutrophils and monocytes, increase in circulation after stroke. The ischemic brain is also recruited to these cells. Encouraged by observed the increase of the proliferation image signal. The hematopoietic progenitor cell activity of the animals with the stroke contributed to an increase in the colony numbers after 7days (14 &15).

Monocytes are characterized by the expression of several clusters of differentiation, such as CD115, CD11c, CD14 and CD16 in human or CD115, CD11b and Ly6C in rodent (16). Interestingly, the current study displayed an apparent increase of CD14 immunostained positive cells in ischemic stroke patients as compared to the control participants. In human, monocytes are regrouped in three main subsets based on their CD14 and CD16 expression levels, which are the classical subset ($CD14^{++}CD16^{-}$), the intermediate subset ($CD14^{++}CD16^{+}$) and the non-classical subset ($CD14^{+}CD16^{++}$) (17).

CD68 is a microglia/macrophage marker related to lysosomal glycoproteins that shuttles in vesicles between lysosomes, endosomes, and the plasma membrane (18). For this reason, various studies (19 & 20), in addition to our results, detected increase in CD68 positive staining in the cytosol oamongf the bone marrow cells. Previous studies have shown that 3-7 days after middle cerebral artery occlusion, blood-derived macrophages were expressed abundantly, even though they were hardly distinguishable from CD68 positive cells (21).

The present findings demonstrate that monocytes increased within two days in the patients with ischemic stroke. Also, the percentages of intermediate monocyte are increased, but non-classical monocyte was decreased, and classical monocyte were unchanged compared to the control. Our results agree with those reported by Urra et al., (2009) (22) and (23), except that for the Kaito et al., study finding that the CD14highCD16- classical Monocyte subtype was increased, which could be attributed to the used monoclonal antibodies in that phenotyping study.

The present study found that the non-classical, CD14lowCD16+ monocyte subpopulation decrease in ischemic stroke patients where they exhibit lower expression levels of Tie2 than other monocyte subsets. Non-classical monocyte elevated in other diseases such as atherosclerosis rheumatoid arthritis, sepsis patients, HIV infection and Patients with active SLE (24; 25 ; 26, 27, 28 & 29). As such, expansion of non-classical monocytes may be a general phenomenon of inflammatory and infectious diseases. However, their role in the manifestations or propagation of these diseases is unclear (30 , 31).

The main finding of the current study is that the expression levels of Tie2 dysregulated on different monocytes subpopulations from ischemic stroke cases as compared to normal subjects (32, 33). The monocytes subsets skewed in stroke patients with expressing higher proportions of angiogenic subset (TEMs) than inflammatory subset (34). TEMs are a subgroup of circulating and tumor-infiltrating myeloid cells with potent proangiogenic activity (35, 36). The examination indicates that the Tie2 expressing monocytes are believed to be integral when it comes to the development of tumor blood vessel as well as emphasize on the possible target to manage tumor angiogenesis and growth (35, 36). The study demonstrates that although TEMs numbers are more than 10-fold higher in the patients that have CLI compared to those in matched controls, with the variance in muscle while significant is less established. Instances of poor limb perfusion occasioned by the consent of critical ischemia could indeed result in curtailing of the TEMs recruitment to the ischemic limb in the CLI patients. Poor limb perfusion is additionally considered accountable for the lack of muscle revascularization despite the instances of increased levels of circulating angiogenic (37).

In addition, it is possible that the TEMs recruited do not survive in the unreceptive environment of the ischemic muscle following the recruitment. It is essential to acknowledge that the increase in circulation of TEMs members could only be linked to the presence of vital ischemia instead of the severity. It was further established that there were no prevalent clinical manifestations correlated with a circulation of TEM (37, 38 ,39).

Data from the prevailing study indicates that TEMs decline into both the CD16+ monocyte subset that was established depending on the intensity of expression of CD14++ CD16+ along with the intermediate CD14++ CD16+ cells. It follows that the intermediate monocyte subset has demonstrated to differentially indicate the high levels of TIE2 and other proangiogenic genes that include endoglin as well as VEGFR2 (40, 41).

For future studies, it is recommended to design functional studies via in vitro angiogenesis assay using tube formation assay. Those studied will evaluate the effect of monocytes from stroke patients on angiogenesis of the brain endothelial cells; and if this will be mediated by Tie2.

Conclusion And Recommendations

In conclusion, the generated data via this study demonstrates the dysregulation of the angiogenic function of monocytes subsets. Their detection in ischemic stroke patients could be an early diagnostic marker to start either a neurovascular repair therapy or improve medications. The new observations act

as the new framework that is used in the process of reevaluating the way global post ischemic inflammation. Especially monocyte subset tends to be differential and time severity dependent. These regulations are connected to stroke pathobiology could allow the development of more creative and efficacious therapeutic strategies.

Declarations

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Institutional Review Board Statement: The study was designed with correspondence to the Ethics Committee of Biomedical Research-Faculty of Medicine at King Abdulaziz University, ethical approval number (Reference No. 237-20, 31 May 2020). The study was executed in consensus with the guidelines followed in King Fahd Center for Medical Research, KAU, Jeddah, Saudi Arabia, which were in accordance with the principles of the declaration of Helsinki.

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data used to support the findings of this study were included within the article.

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Author Contribution: A.A.: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation, Visualization, Investigation, Supervision, Software, Validation, Writing- Reviewing and Editing.

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Figures

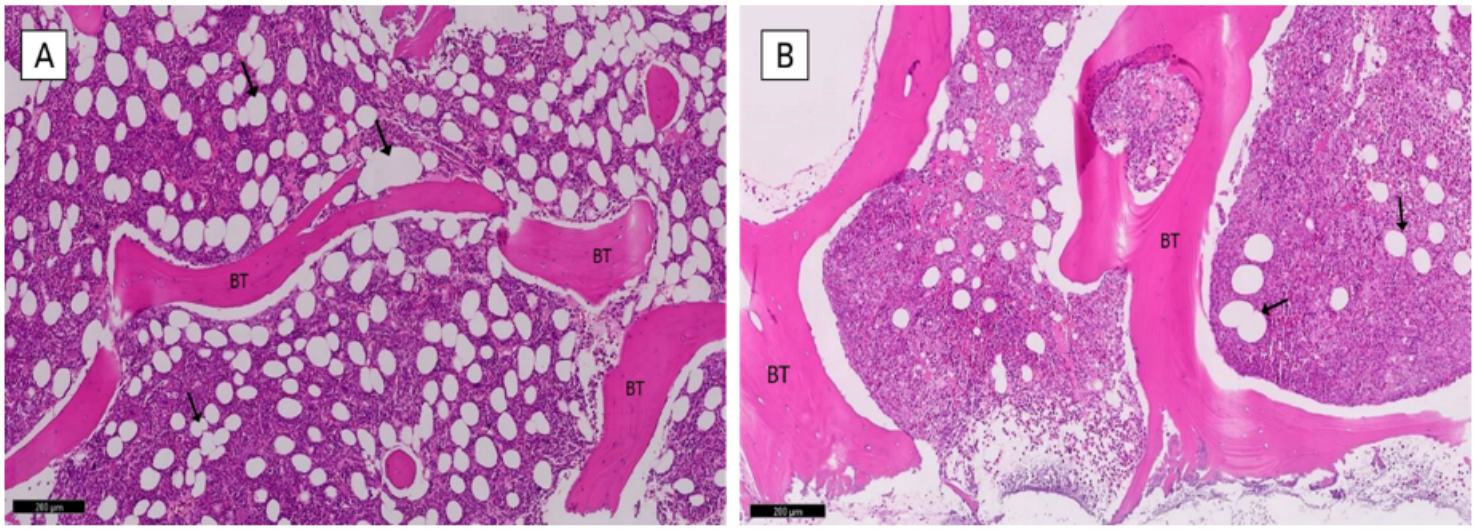

Figure 1

Photomicrographs of the H& E section of the bone marrow trephine biopsy showing:

A: control subject with bone trabeculae (BT) radiating between it bone marrow cells, blood sinusoids and adipose tissue (↑). Scale bar 200 μm

B: Ischemic stroke patient case has hypercellular marrow between bone trabeculae (BT) with apparent decrease of the adipose tissue (↑). Scale bar 200 μm

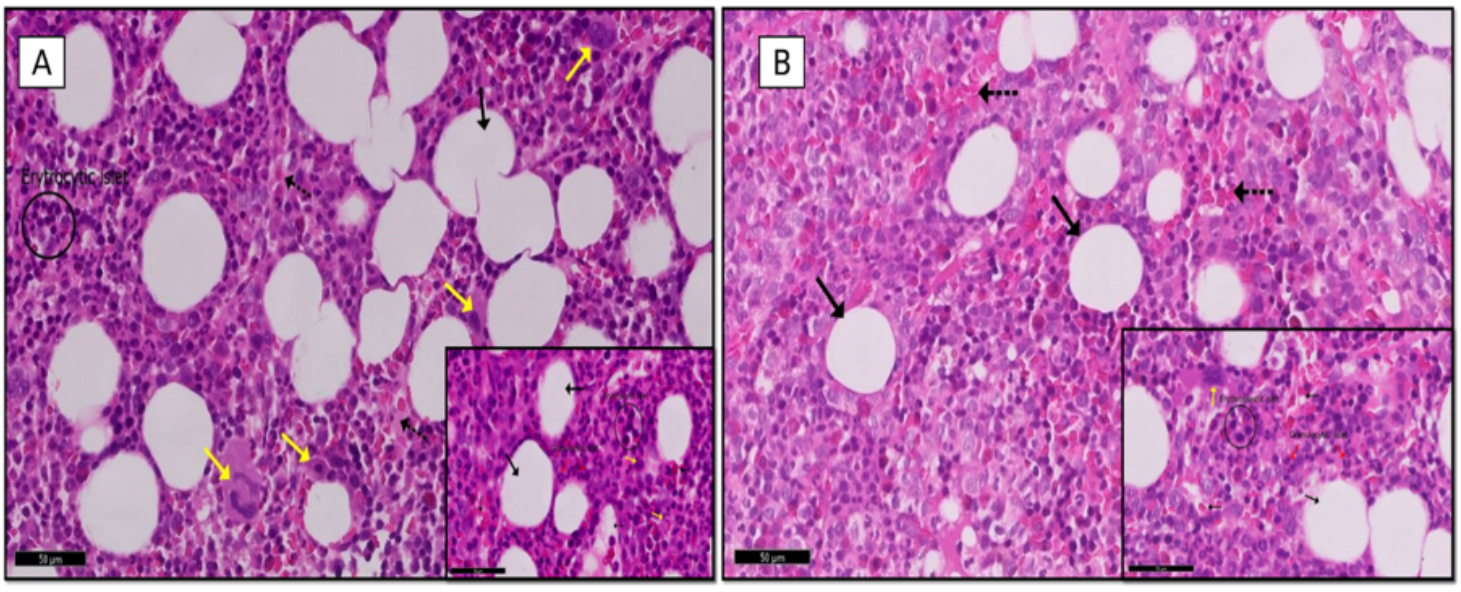

Figure 2

Photomicrographs from the H&E section from the bone marrow trephine biopsy showing:

A: a control subject, with apparent increase of the adipose tissue (↑) and in-between blood sinusoid (dot arrow) compared to the cellular elements. Scale bar 50 μm. Inset: higher magnification showing

distribution of the erythroid series, granulocyte series (red arrow), and megakaryocytes (yellow arrow). Scale bar 50 μm

B: An ischemic stroke patient hypercellular marrow with apparent decrease of the adipose tissue (↑) and an apparent increase of dilated blood sinusoids (dot arrow). Scale bar 50 μm Inset: higher magnification showing erythroid series and an apparent increase of granulocyte series (red arrow) with megakaryocytes (yellow arrow). Scale bar 50 μm

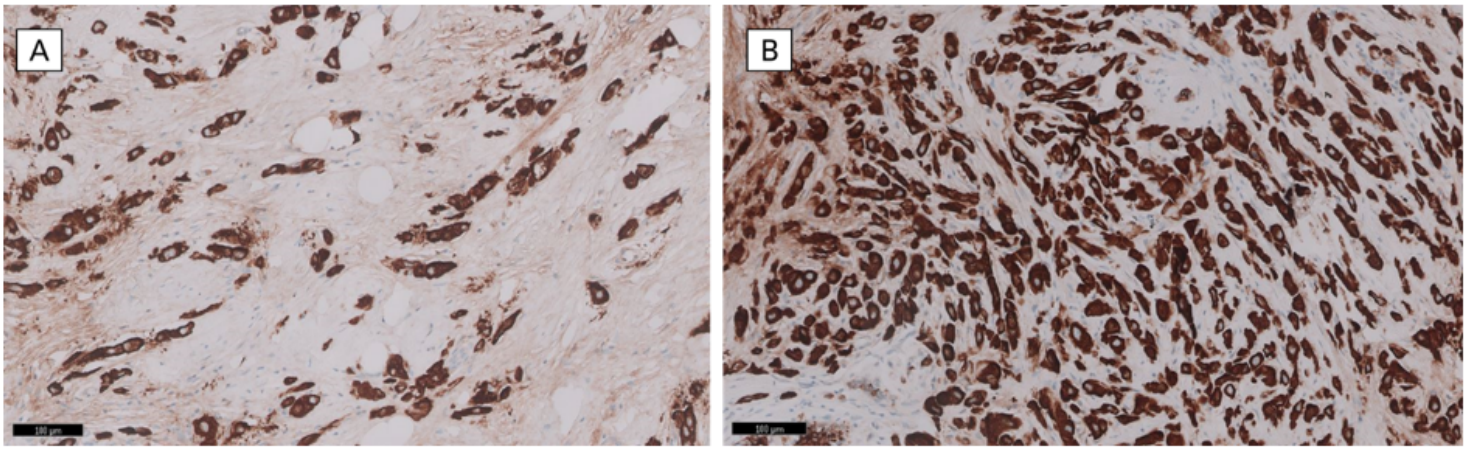

Figure 3

Photomicrographs from sections of the bone marrow trephine biopsy immunostained by CD14 showing:

A: the sparce loose clusters of the CD14 the brown stained immunopositive cells in control patient. Scale bar 100 μm

B: an apparent increase in the clusters of the CD14 brown stained immunopositive cells in ischemic stroke patient. Scale bar 100 μm

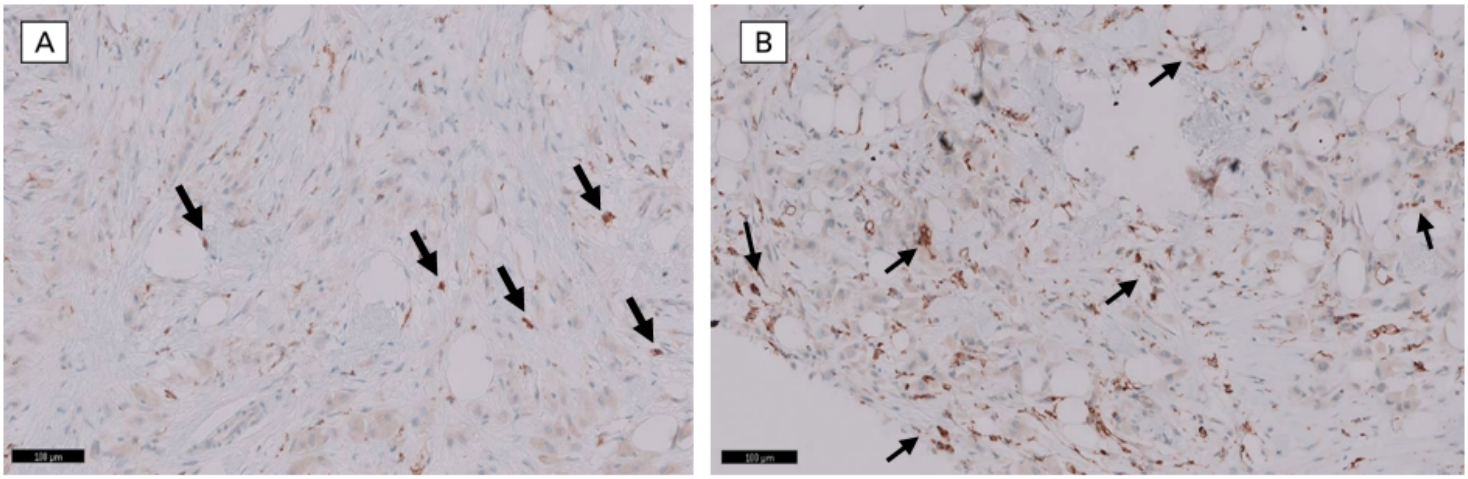

Figure 4

Photomicrographs from sections of the bone marrow trephine biopsy immunostained by CD68 showing:

A: A control subject has a sparce number of the brown stained CD68 immunopositive cells (→). Scale bar 100 µm

B: An ischemic stroke patient section an apparent increase in the brown stained CD68 immunopositive cells in. Scale bar 100 µm

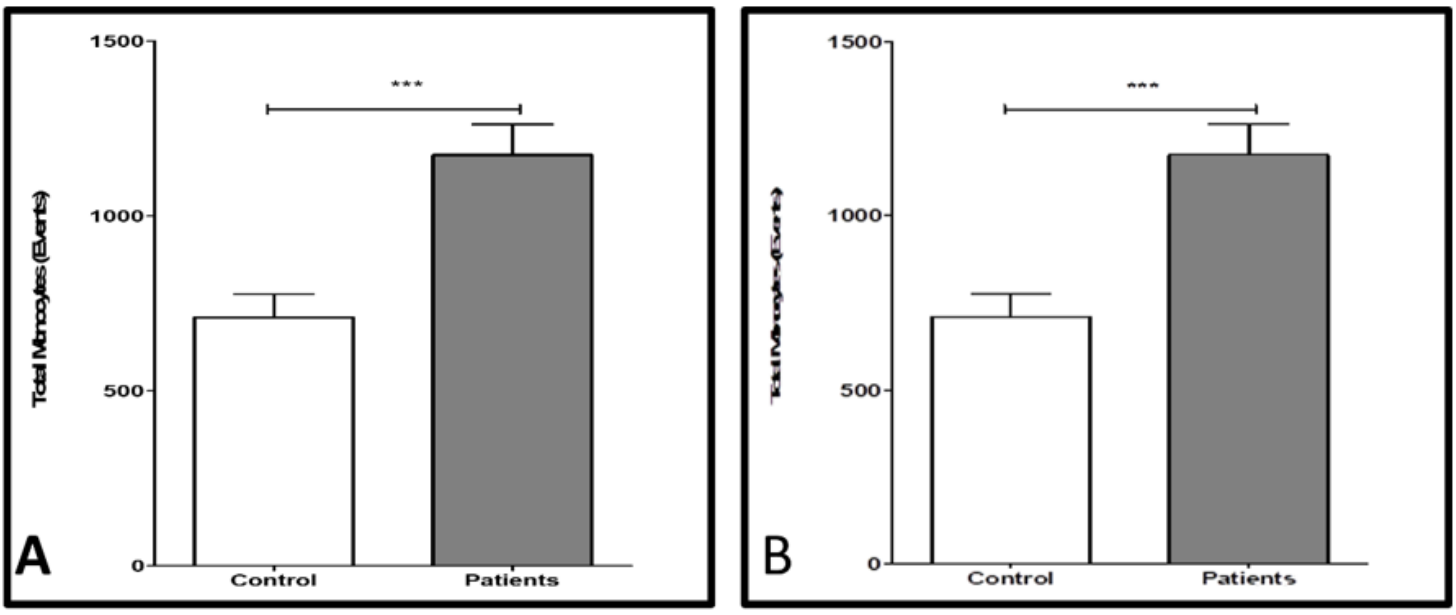

Figure 5

Comparison between patients and control of A. Total Leukocytes (Events) level and B. Total monocytes (Events) level**.** Data are expressed as mean +/- standard error. Significant was made using unpaired student “t” test.

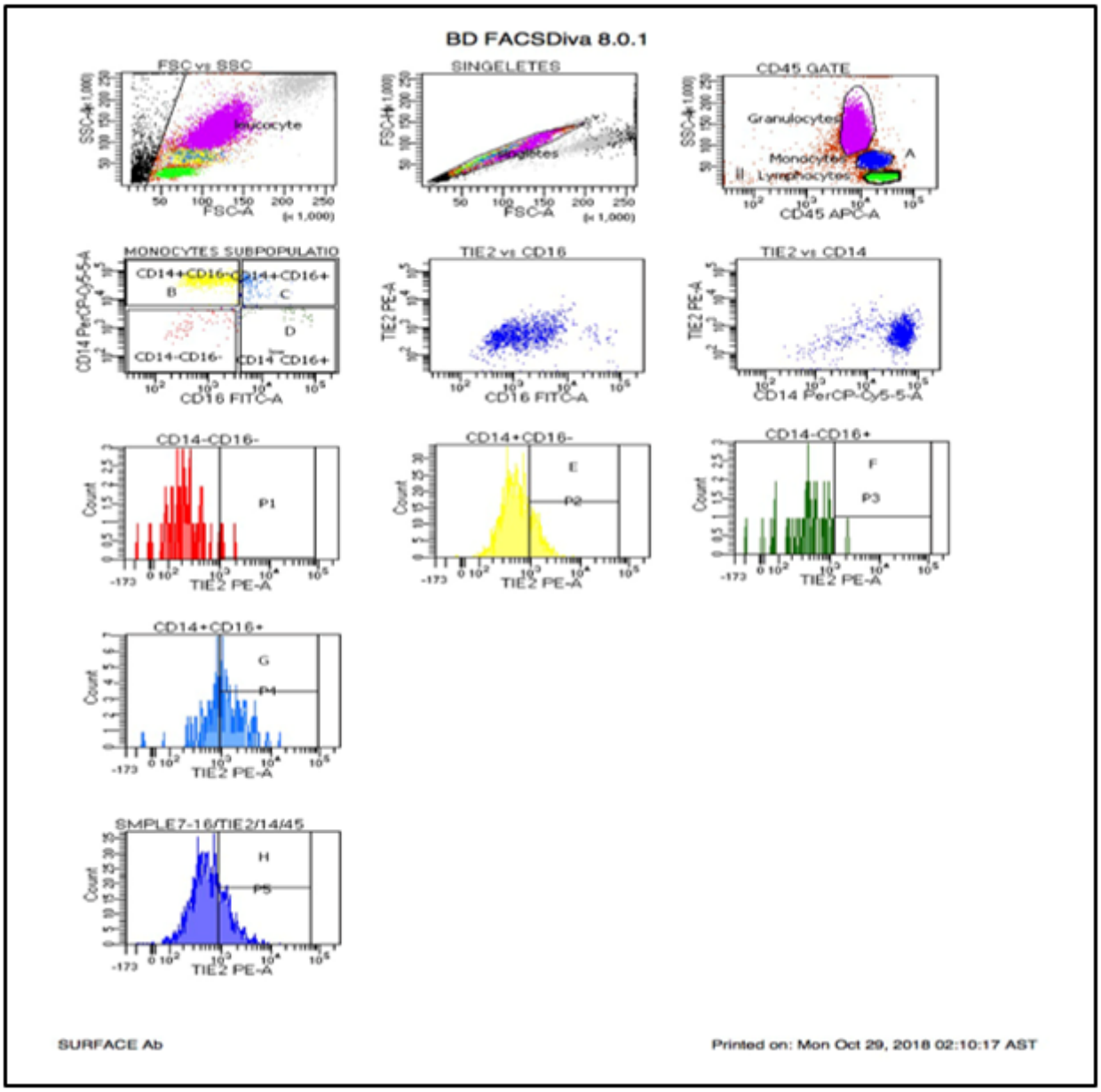

Figure 6

Gating strategy used in flow cytometry analysis to detect different subsets of monocytes. Monocytes identification as CD45+/SSC intermediate cells **(A)**. Characterization of human monocyte subsets into CD14+/CD16− (classical), CD14+/CD16+ (intermediate), and CD14low/CD16+ (non-classical) subsets **(B-D)**. TIE2 expression on classical monocyte**(E)**. TIE2 expression on intermediate monocyte(**F**). TIE2 expression on non-classical monocyte(**G**). TIE2 expression on total monocyte (**H**).

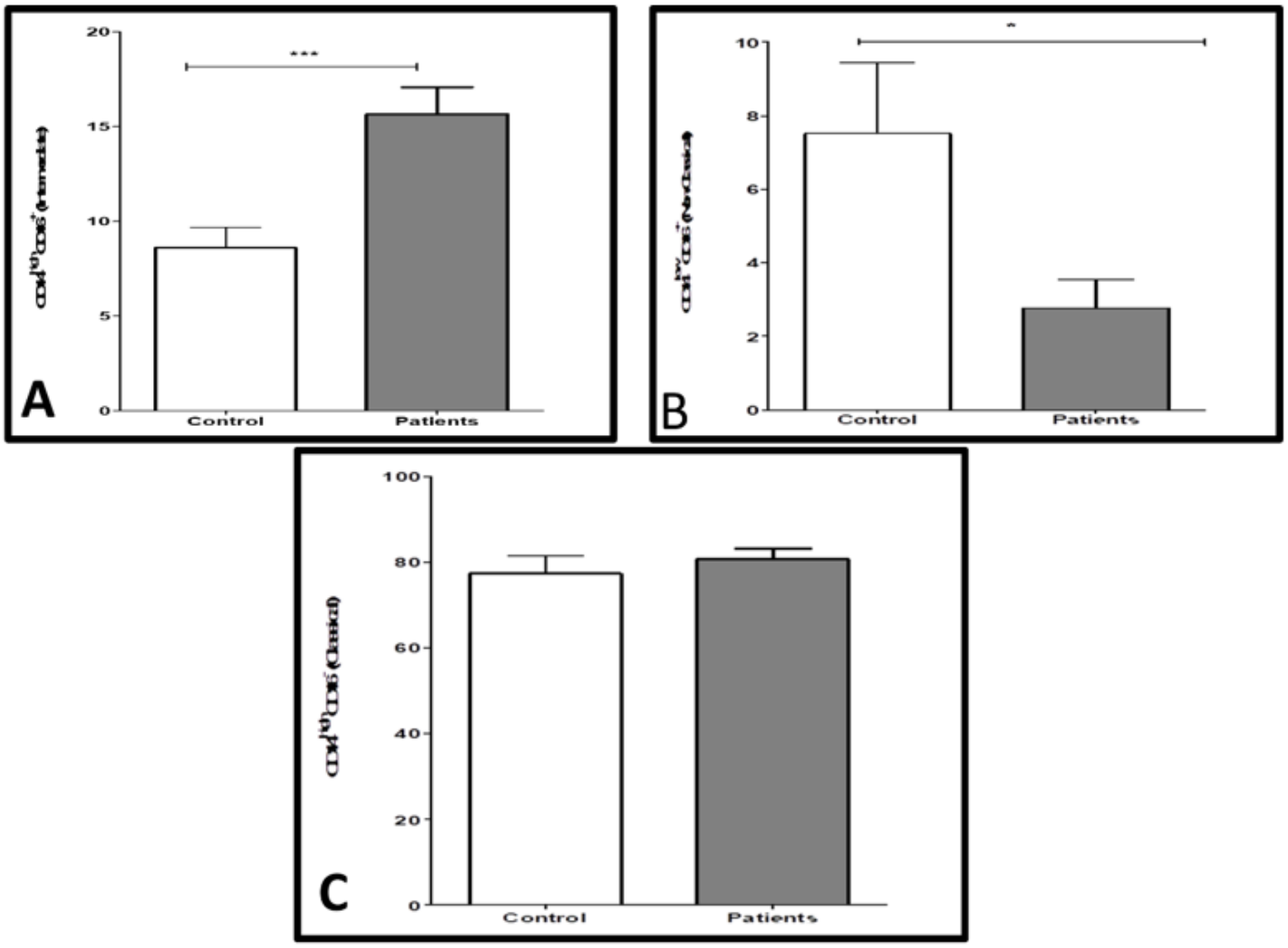

Figure 7

Comparison between patients and control regarding **A**. $CD14^{high}CD16^{+}$ (Intermediate) level**. B.** $CD14^{low}CD16^{+}$ (Non classical). **C.** $CD14^{high}CD16^{+}$ (Classical) level Data are expressed as mean +/- standard error. Significant was made using unpaired student “t” test.

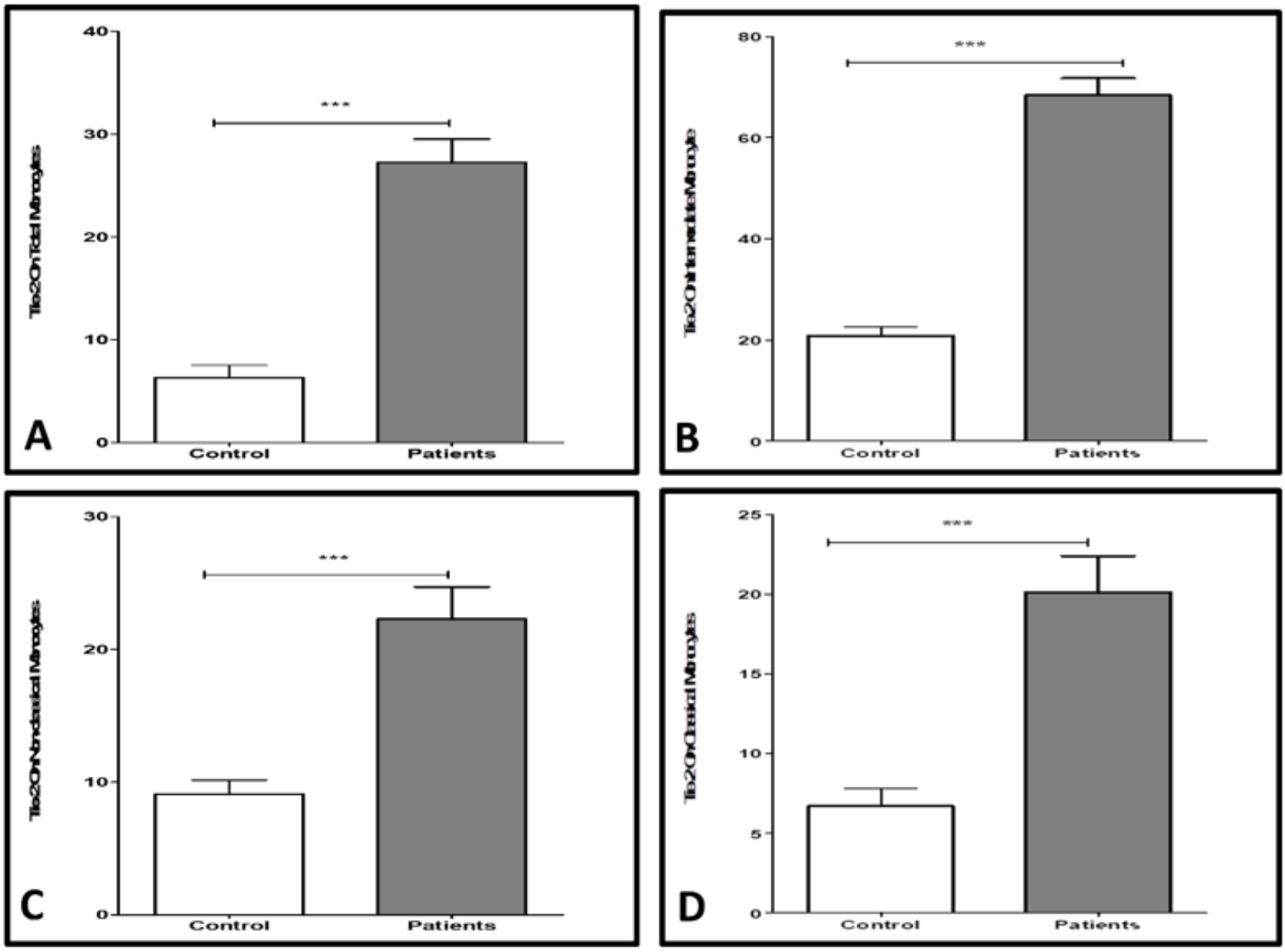

Figure 8

Comparison between patients and control regarding A. Tie-2 On Total Monocytes level. B. Tie-2 On Intermediate Monocyte level**. C.** Tie-2 On Non-classical Monocytes level**. D.** Tie-2 On classical Monocytes level. Data are expressed as mean +/- standard error. Significant was made using unpaired student “t” test.

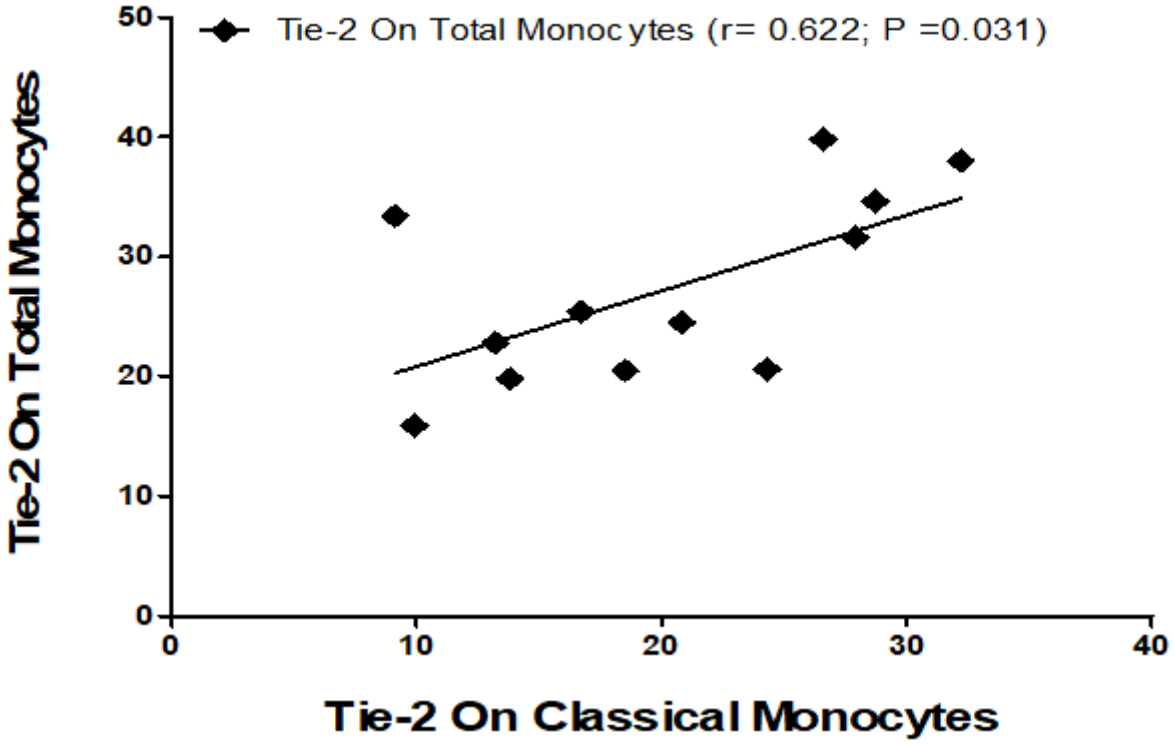

Figure 9

Correlations coefficient between Tie-2 On Classical Monocytes and Tie-2 On Total Monocytes in patients (r= 0.622; P > 0.05) using Person test.