

## Phenotype Changes of Circulating Monocytes in a Hypercholesterolemic Swine Model of Coronary Artery Disease

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### Abstract

**Objective:** Inflammation and immunity activation play a key role in atherosclerosis (ATS) onset and progression. Aim of this study was to investigate the relationships between phenotype of circulating monocytes and coronary artery disease (CAD) development in a histologically well-characterized swine model of ATS.

**Methods:** Blood samples were obtained from 6 animals at baseline and after 16 weeks high fat cholesterol-enriched diet. Flow cytometry monocyte identification was performed (CD172a marker). Adhesion (CD18a, CD11a, CD11R3, CD49d, CD29), differentiation (CD14) and activation receptors (SLA-DR, CD16, CD163) were quantified as percentage of positivity (%) and Relative Fluorescence Intensity (RFI). Lipid parameters (LDL, oxLDL, HDL) and soluble endothelial ICAM-1 were measured and histologic quantitative assessment of coronary ATS was performed.

**Results:** Flow cytometry analysis demonstrated a significant post-diet decrease of CD14 RFI and an increment of % SLA-DR. Pre-diet values of ICAM-1 and % SLA-DR correlated reciprocally ( $P=0.0191$ ) and with several CAD severity indexes ( $P\leq 0.02$ ). Positive correlations between RFI changes of CD29 ( $P=0.0213$ ) and CD18a ( $P=0.0341$ ) and morphometric indexes of coronary ATS were found. Post-diet RFI values of CD29, CD18a and CD16 were also closely related to morphometric parameters ( $P<0.03$ ). A cumulative post-diet tendency to increase of CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction ( $45.07 \pm 2.27$  vs.  $40.14 \pm 3.16$ ) and a tight correlation between changes of this monocyte subset and corresponding HDL variations ( $P=0.0100$ ) were also observed.

**Conclusions:** Blood monocyte orientation towards a macrophage-like phenotype, similar to a HDL-induced maturation, and a close association between markers changes and severity of diet induced coronary ATS could provide new insights into plaque growth and progression in CAD.

**Keywords:** Monocyte; Swine; Peripheral blood; Flow cytometry; Hypercholesterolemic diet; Coronary atherosclerosis; Histopathology

### Abbreviations:

ATS: Atherosclerosis; HCD: Hypercholesterolemic Diet; CAD: Coronary Artery Disease

### Introduction

Atherosclerosis (ATS) is a chronic inflammatory process of large and medium-size arteries, in which the focal endothelial activation, induced by hypercholesterolemia and lipoproteins deposition in the arterial intima, represents an essential initiating event [1,2]. The endothelial activation determines an increased adhesion molecule expression and chemokine production, which mediate the initial rolling of blood leukocytes on the endothelial vascular surface, their subsequent firm adhesion at activation sites, and their final recruitment into the underlying intima [3,4]. Moreover, hemodynamic flow patterns, typical of ATS-prone segments (low or oscillatory shear stress), give rise to an increased expression of adhesion molecule and inflammatory genes by endothelial cells and, together with lipoprotein

accumulation, aggravate intimal inflammation [5]. Among blood leukocyte subsets, the study of monocyte migration and monocyte-derived macrophage accumulation into arterial wall, in both early atherogenesis and advanced plaque progression, has become a topic of great interest in the last decade [6-8]. In particular, immunohistochemical analysis of human atherosclerotic lesions, as well as experimental studies in gene-targeted mice, suggested that the monocyte integrins VLA-4 (very late antigen-4) and LFA-1 (lymphocyte function-associated antigen-1) and their corresponding endothelial cell ligands, VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1), are particularly important in plaque initiation [7]. Furthermore, the interest concerning the role of different monocyte subsets in ATS initiation and progression has arisen due to the demonstration of phenotypic and functional heterogeneity of circulating monocytes in mice, humans and other species [8-10]. Two major subsets of blood monocytes have been described based on differential expression of CCR1, CCR2 and CX3CR1 (fractalkine receptor) chemokine receptors [11]. The two murine monocyte subsets, classical (referred as Ly6C<sup>high</sup>) and non-classical (referred as Ly6C<sup>low</sup>) are homologous to the human classical (CD14<sup>high</sup>/CD16<sup>neg</sup>) and non-classical (CD14<sup>low</sup>/CD16<sup>high</sup>) monocyte subsets [10]. In hypercholesterolemic mice the

monocytosis is dominated by the classical subset and correlates with atherosclerotic plaque size and foam cell content [12]. However, there are important differences in the characterization and role of monocyte subset and function in humans as compared to mice. Therefore, the choice of the animal model that more accurately reflects the process of atherogenesis in humans is mandatory. With the completion of the genome sequence and the characterization of many regulatory molecules and markers, the pig has emerged as a predictive experimental model for the study of human innate immunity in several inflammatory pathologies, overcoming the limitations of data derived from rodents [13]. A previous immunohistochemical study demonstrated the translational potential towards human pathology of a swine model of vascular damage associated with mild hypercholesterolemia [14]. Other histological studies in pigs showed that experimentally induced hypercholesterolemia produces coronary endothelial dysfunction with intimal hyperplasia by accumulation of lipids and foam cells in the vascular wall [15,16]. Moreover, atherogenic diet has been shown to increase the level of cholesterol efflux transporter ABCA1 in pig blood monocytes prior to atherosclerotic lesion formation, and higher ABCA1 levels are associated with higher plaque complexity [17].

The aim of this study has been to evaluate by flow cytometry the phenotype changes of circulating monocytes in a porcine model of coronary artery disease (CAD) induced by a hypercholesterolemic diet (HCD) and to investigate, for the first time, their relationships with the degree of coronary ATS development measured by comprehensive histomorphometric analysis.

## Materials and Methods

### Animals and Diet

Six male domestic pigs (mean body weight  $36 \pm 2$  Kg at baseline) were purchased from a commercial breeder. Animal instrumentation and experimental protocol were approved by the Animal Care Committee of the Italian Ministry of Health and were in accordance with the Italian Law (DL-116, Jan. 27, 1992), which is in compliance with the National Institute of Health publication *Guide for the Care and Use of Laboratory Animals*. Pigs were fed for 4 months with a high-lipid diet containing lard (20%), cholesterol (4%), oil (3%), fibers (7%), sodium cholate (1.5%), besides proteins (17.5%) and carbohydrates (33%). The total energy from the HCD was 4450 Kcal/Kg with a fatty energy fraction corresponding to 2430 Kcal/Kg (54.6% of the total energy contribution). Both at baseline (pre-diet) and at the end of the study (post-diet), each animal underwent blood sampling for monocyte flow cytometry analysis. At the end of diet period, animals were sacrificed under anaesthesia (intramuscular administration of 10mg/kg of Zoletil®, 0.05 mg/kg atropine and maintained with isoflurane, together with 5 mg/kg/hour of propofol intravenous infusion); the whole heart was harvested for histology and immunohistochemistry processing of coronary arteries.

### Histology and Morphometry

Consecutive coronary segments, 0.5 cm thick, were harvested from the left (LMCA, LAD, LCX) and right coronary (RCA) arteries of each animal. Following standard histology processing, 20-30 serial cross-sections, 5  $\mu$ m thick, were cut from paraffin embedded samples by rotary microtome (Microm HM 300, Bio-optica).

Consecutive sections of each arterial segment were stained with both standard (Haematoxylin and Eosin, H&E) and with Masson trichrome and Weigert van Gieson (WVG) stains for collagen and elastic laminae respectively, and examined under light microscopy (Olympus BX43) at 4X to 40X original magnification. After acquisition by a video system (Olympus D70 camera) interfaced to a computer with dedicated software (Olympus Cell Sens Dimension), image processing and morphometric analysis of each section were performed.

All lesions were classified from type I to VI, according to the current Stary's stages for atherosclerotic plaques of the American Heart Association Committee on Vascular Lesions [18].

Lesion size was expressed in terms of thickness and cross-sectional area. The standard morphometric parameters used to define plaque thickness are the maximal distance from internal elastic lamina to endothelial layer (maximal intimal thickness, IT) and the maximal intima to media thickness ratio (IMT ratio); for plaque area, as coronary cross sections are affected by histologic distortion and shrinkage of the wall [19], we used, in addition to absolute lesion area value (LA, mm<sup>2</sup>), the ratio of lesion to total vessel wall area (intima +media area surrounded by external elastic lamina) $\times$ 100 (% lesion area, % LA). All four indexes were calculated in each section and used as a quantitative estimate of overall ATS burden by averaging the values of all sections/segments which display pathologic changes in each animal and expressed as Mean IT, Mean IMT ratio, Mean LA and Mean% LA. The most severe plaque of each case was taken as a marker of maximal coronary ATS progression and its parameters measured and expressed as Max IT, Max IMT ratio, Max LA and Max% LA. The relative percentage of atheromas (Frequency of Atheromas) (Stary's stage IV or higher) on the total number of lesional segments in each case was also assessed. The number of coronary segments analyzed for quantitative assessment of CAD and the incidence of distinct lesion types according to Stary's stages were summarized in Table 1.

	N.	%
All coronary Segments	135	100
Segments with lesions	113	84
Type I	18	16
Type II	45	40
Type III	14	12
Type IV	11	10
Type Va	14	12
Type Vb	11	10
HCD:Hypercholesterolemic Diet		

**Table 1:**Total number of coronary segments analyzed and incidence (number, N. and percentage, %) of each lesion type in the 6 HCD-treated pigs.

### Blood sampling

Blood samples were collected at baseline and at the post-diet time points. Blood cell counts were performed on EDTA-anticoagulated samples by the ADVIA 2120 White Blood Cell Technology System (Siemens Healthcare Diagnostics, Italy) to determine red blood cells

(RBCs), platelets (PLTs) and total white blood cells (WBCs) counts, as well as hemoglobin (Hb) and hematocrit (Hct) levels. Blood smears were May-Grunwald-Giemsa stained and visually evaluated for white blood cell differential counts. The plasma level of high-density lipoprotein (HDL) was quantified by standard enzymatic techniques (Synchron CX9 Pro, Beckman Coulter, Inc., Fullerton, CA, USA). Low-density lipoproteins (LDL) were quantified according to Friedewald formula [20]. Aliquots of plasma obtained from EDTA-anticoagulated blood samples centrifuged at 3,000g for 10 min at 4°C, and immediately frozen at -80°C, were used to detect and quantify by ELISA tests the circulating levels of pro- and anti-inflammatory cytokines (IL-6, TNF- $\alpha$ , IFN- $\gamma$ , IL-10) and oxidized low-density lipoproteins (oxLDL), as well as of the endothelial adhesion molecule ICAM-1. The minimum detectable concentrations for each cytokine were 3.7 pg/mL for TNF- $\alpha$ , 2.03 pg/mL for IL-6, 6.01 pg/mL for IFN- $\gamma$  and 3.5 pg/mL for IL-10.

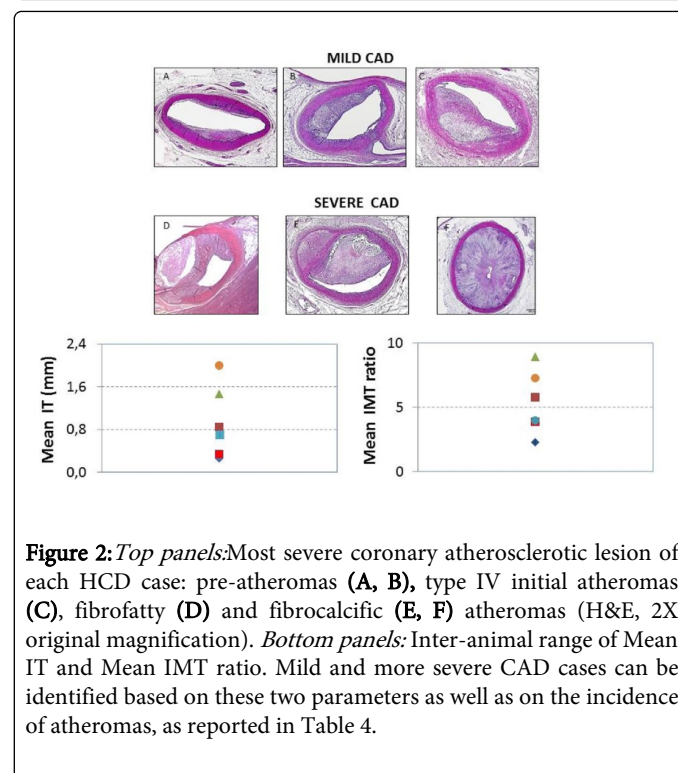
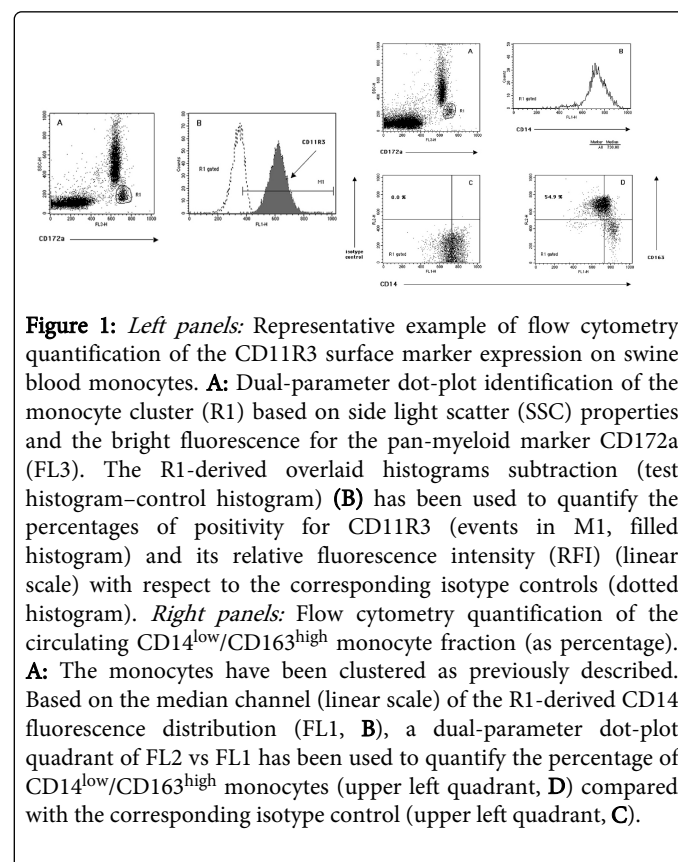
### Flow cytometry analysis

The evaluated swine monocyte surface markers were: CD172a, CD14, SLA-DR, CD16, CD163, CD18a, CD11a, CD11R3, CD49d, CD29.

Flow cytometry analysis of swine blood monocytes was carried out on EDTA-anticoagulated samples, according to a triple staining procedure already applied by our group to a phenotypical characterization of human circulating monocytes during extracorporeal circulation [21,22]. Briefly, after red blood cells lysis using an ammonium-chloride based solution, the isolated leukocyte suspension were treated as follows: a) primary on ice incubation for 30 min with a purified mouse monoclonal antibody (IgG1 isotype) recognizing the swine pan-myeloid marker CD172a (SWC3); b) washing with cold PBS (BSA 0.5%) saline solution (staining buffer) by centrifugation at 300g for 10 min at 4°C to eliminate the unbound antibody; c) on ice incubation for further 30 min with a Peridinin Chlorophyll Protein (PerCP)-conjugated rat anti-mouse IgG1 secondary antibody; d) washing and subsequent double staining on ice for 30 min with a mixtures of Fluorescein Isothiocyanate (FITC)- and Phycoerythrin (PE)-conjugated mouse monoclonal antibodies directed against the porcine monocyte surface molecule combinations CD11a/CD18a, CD11R3/CD18a, CD49d/CD29, CD14/CD16, CD14/CD163, SLA-DR/CD163; e) final washing and re-suspension in 400  $\mu$ l of cold staining buffer for flow analysis. Mouse isotype controls were carried out in parallel to quantify the unspecific binding of the main antibodies. Monoclonal antibodies were obtained from Immunotech (Beckman Coulter, Fullerton, CA, USA), Novus Biologicals (Littleton, CO, USA), Serotec (MorphoSys Ltd, Kidlington, UK) and R&D Systems (Minneapolis, MN, USA). The flow cytometry acquisition process was stopped after 5,000 monocytes, simultaneously gated on the basis of their side scatter (SSC) properties and their bright staining for CD172a (FL3), were collected for each sample (Figure 1).

Flow data analysis was based either on measurement of positive events for each marker (as percentage) or on quantification of its relative amount per cell, in terms of relative fluorescence intensity (RFI), expressed as linear units after subtracting the median value of the isotype-matched control histogram from that corresponding to the specific marker (Figure 1). Moreover, a dual parameter dot-plot of FL2 (CD163) vs. FL1 (CD14) was used to quantify (as percentage) the CD14<sup>low</sup>/CD163<sup>high</sup> blood monocyte fraction (Figure 2), as reported [13]. Flow cytometry analysis was performed with a FACScan

instrument and the CellQuest software program (Becton Dickinson, San Jose, CA, USA).



### Statistical analysis

The mean values of measurements, their standard errors (SEM) and standard deviations (SD), as well as the linear regressions among different groups of data were calculated with the Stat View 5.0 software program (SAS Institute, Cary, NC, USA). The statistical significant differences between groups was determined by Fisher's t-test for repeated measurements. A  $P < 0.05$  was considered statistically significant.

### Results

#### Hypercholesterolemic diet (HCD)-induced biochemical changes and hematological modifications

The animal weight ranged from  $30.833 \pm 1.939$  at baseline to  $49.167 \pm 3.081$  kg after 16 weeks HCD ( $P < 0.0001$ ). There was a significant

post-diet increase of total cholesterol and LDL concentrations compared with baseline values. Post-diet vs. baseline increments of HDL and oxLDL concentrations, although statistically not significant, were also observed.

An augmentation, even if statistically not significant, of post-diet vs. baseline blood levels of the pro-inflammatory cytokines TNF- $\alpha$  and IL-6 has been observed. The circulating levels of cytokines IFN- $\gamma$  and IL-10 resulted undetectable, while sICAM-1 level was not significantly changed.

HCD-treated pigs exhibited a slight post-diet decrease of total WBCs count and hematocrit volume compared with baseline values. These findings were associated to a not significant post-diet decrease of RBC counts ( $n^\circ$  of cells  $\times 10^6/\mu\text{L}$ ) ( $5.083 \pm 0.192$  vs.  $5.755 \pm 0.292$ ,  $P = 0.0507$ ) and blood monocyte number ( $n^\circ$  of cells/ $\mu\text{L}$ ) ( $850 \pm 99$  vs.  $1060 \pm 183$ ,  $P = \text{ns}$ ). Table 2 summarizes these results.

	Baseline	Post-diet	P
WBC(*1000 n/ $\mu\text{L}$ )	12.98 $\pm$ 6.31	10.26 $\pm$ 2.49	ns
HCT %	27.72 $\pm$ 3.91	25.08 $\pm$ 1.16	ns
Creatinine (mg/dL)	0.99 $\pm$ 0.32	1.78 $\pm$ 0.57	0.0220
Glucose (mg/dL)	90.00 $\pm$ 25.59	67.17 $\pm$ 31.73	ns
Total Cholesterol (mg/dL)	71.83 $\pm$ 29.03	557.83 $\pm$ 133.73	0.0001
HDL (mg/dL)	24.83 $\pm$ 12.54	33.67 $\pm$ 14.35	ns
LDL (mg/dL)	35.10 $\pm$ 17.27	511.17 $\pm$ 133.60	0.0002
Ch tot/HDL ratio	3.42 $\pm$ 0.65	18.88 $\pm$ 7.08	0.0129
oxLDL (U/L)	17.75 $\pm$ 6.69	45.50 $\pm$ 43.99	ns
TG (mg/dL)	59.00 $\pm$ 50.05	65.17 $\pm$ 53.94	ns
GOT (IU/L)	39.50 $\pm$ 29.95	112.00 $\pm$ 113.27	ns
GPT (IU/L)	31.33 $\pm$ 6.74	47.83 $\pm$ 8.59	0.0314
Gammaglutamyl-transferase (UI/L)	33.50 $\pm$ 16.81	74.40 $\pm$ 25.15	0.0368
Apolipoprotein A-I (mg/dL)	25.96 $\pm$ 12.97	54.65 $\pm$ 6.37	0.0039
sICAM-1 (ng/mL)	567.88 $\pm$ 330.30	503.61 $\pm$ 175.47	ns
IL6 (pg/mL)	89.35 $\pm$ 122.68	128.36 $\pm$ 142.21	ns
TNFalpha (pg/mL)	55.40 $\pm$ 28.83	405.30 $\pm$ 847.44	ns

HCD:Hypercholesterolemic Diet;SD:Standard Deviation;ns: not significant

**Table 2:** Cumulative quantitative variations (Mean  $\pm$  SD) of biochemical and hematological parameters in the 6 HCD-treated pigs.

#### Phenotype changes of circulating monocytes after 16 weeks HCD

The porcine mononuclear phagocyte system, as the human system, is characterized by the expression of several surface molecules [23]. The modulation of these phenotype markers identifies different levels of cellular activation and functional capabilities [24,25].

The cumulative analysis of blood monocyte surface markers revealed a significant post-diet down modulation of CD14 expression,

either as percentage of positivity or relative fluorescence intensity (RFI) (Table 2). Moreover, compared with baseline values, a post-diet increase of SLA-DR expression significant in terms of cell positive fraction, was also evident (Table 3).

The post-diet mean value of the CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction (as percentage) was found to be slightly higher in comparison with baseline data ( $45.07 \pm 2.27$  vs.  $40.14 \pm 3.16$ ,  $P = \text{ns}$ ).

Marker	%Positivity (Baseline)	% Positivity (Post-diet)	RFI (Baseline)	RFI (Post-diet)	Functional description
CD14	99.25 ± 0.228	97.87 ± 0.516 (P=0.0083)	490.75 ± 10.063	428.91 ± 16.192 (P=0.0128)	Monocyte differentiation/maturation antigen
CD18a	99.99 ± 0.008	99.93 ± 0.058 (P=ns)	628.25 ± 10.446	638.25 ± 14.631 (P=ns)	β chain of the leukocyte integrin complexes CD11a/CD18 (LFA-1) [ligand for ICAM-1, ICAM-2, ICAM-3 on endothelial cells] and CD11b/CD18 (Mac-1) [ligand for ICAM-1 on endothelial cells]
CD11a	99.83 ± 0.036	99.43 ± 0.385 (P=ns)	412.41 ± 7.200	390.16 ± 8.324 (P=ns)	α chain of the leukocyte integrin complex LFA-1
CD11R3	98.98 ± 0.210	99.01 ± 0.229 (P=ns)	323.33 ± 13.803	350.00 ± 17.025 (P=ns)	α chain of the leukocyte integrin complex Mac-1, equivalent to human CD11b
CD49d	99.09 ± 0.189	99.09 ± 0.217 (P=ns)	323.50 ± 6.136	305.33 ± 5.829 (P=ns)	α chain of the leukocyte integrin complex CD49d/CD29 (VLA-4) [ligand for VCAM-1 on endothelial cells]
CD29	99.96 ± 0.013	99.93 ± 0.016 (P=ns)	674.33 ± 12.393	674.75 ± 24.605 (P=ns)	β chain of the leukocyte integrin complex VLA-4
CD16	99.98 ± 0.010	99.96 ± 0.018 (P=ns)	585.16 ± 17.155	595.25 ± 14.180 (P=ns)	FcγRIII, low affinity receptor for IgG
CD163	99.94 ± 0.031	99.20 ± 0.475 (P=ns)	581.27 ± 42.086	605.45 ± 33.004 (P=ns)	Haptoglobin scavenger receptor
SLA-DR	48.26 ± 2.336	62.06 ± 5.432 (P=0.0379)	249.75 ± 14.156	284.41 ± 20.086 (P=ns)	Monocyte activation marker, equivalent to human HLA-DR

HCD:Hypercholesterolemic Diet;SEM, Standard Error of the Mean;ns: not significant

**Table 3:** Cumulative quantitative variations (Mean ± SEM) of surface markers expression (as percentage of positivity and RFI) in the 6 HCD-treated pigs. Functional description of the markers is reported.

### Histology and histomorphometry

The majority of coronary samples (84%) displayed atherosclerotic changes: out of the 113 coronary segments with coronary lesions, type IV (atheroma) to type V plaques were present in about one third, while type III pre-atheroma or earlier stages of pre-atherosclerotic lesions in the other ones.

Histomorphometry	HCD cases(6)	MILD CAD (3)	SEVERE CAD (3)
%Atheromas	28.67 ± 17.23	9.00 ± 6.14	48.33 ± 20.36
Mean IT (mm)	0.59 ± 0.30	0.36 ± 0.11	0.84 ± 0.25
Mean LA (mm <sup>2</sup> )	2.40 ± 1.24	1.03 ± 0.20	2.85 ± 1.14
Mean IMT ratio	2.64 ± 1.40	1.19 ± 0.36	3.49 ± 1.11
Mean LA%	31.17 ± 8.25	19.78 ± 6.11	42.58 ± 7.60

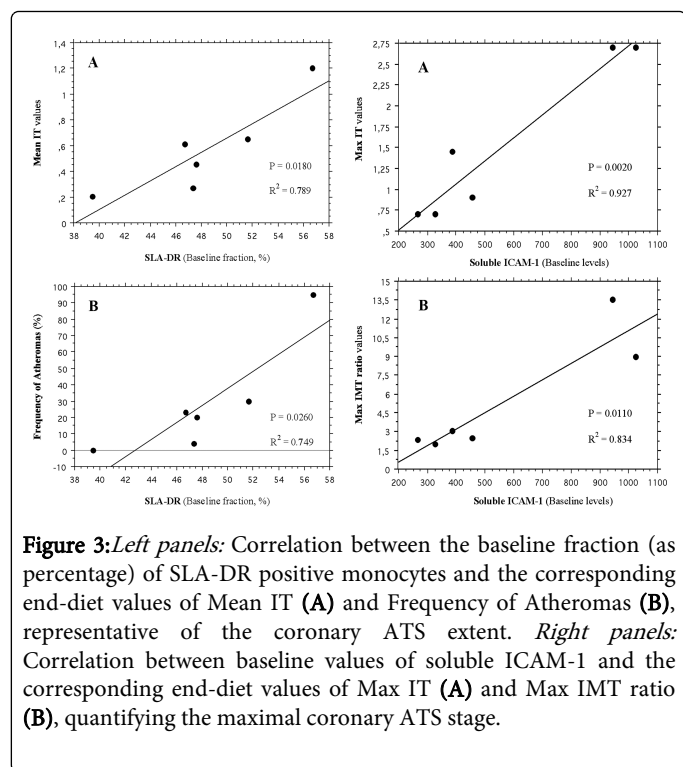
SEM: Standard Error of the Mean; CAD: Coronary Artery Disease

**Table 4:** Mean values ± SEM of most relevant histomorphometric parameters in all cases as well as in the two separate subgroups with different CAD severity.

Top panels of Figure 2 illustrate the spectrum of lesions (maximal lesion of each case) observed in six HCD treated animals. Histomorphometry of coronary lesions confirmed a broad inter-animal variability in lesion stage and severity. Mild (3 cases) and more severe (3 cases) coronary ATS is observed in HCD treated animals and a lower variability of ATS burden found in each subgroup, as summarized in Table 4 for the relevant morphometric parameters measured.

### Associations between monocyte phenotype changes and histomorphometric parameters of ATS burden

The pre-diet activation state of circulating monocytes, represented by the fraction (percentage) of cells expressing the activation marker SLA-DR (with a CV, coefficient of variation=12%), significantly correlated with the end-diet values of histopathologic parameters used to quantify coronary ATS spreading, such as Mean IT, and stage of progression (Frequency of Atheromas), as depicted in Figure 3 (left panels). The monocyte percentages of positivity for SLA-DR correlated positively with baseline values of soluble ICAM-1 (P=0.0191, R<sup>2</sup>=0.783).



**Figure 3:** Left panels: Correlation between the baseline fraction (as percentage) of SLA-DR positive monocytes and the corresponding end-diet values of Mean IT (A) and Frequency of Atheromas (B), representative of the coronary ATS extent. Right panels: Correlation between baseline values of soluble ICAM-1 and the corresponding end-diet values of Max IT (A) and Max IMT ratio (B), quantifying the maximal coronary ATS stage.

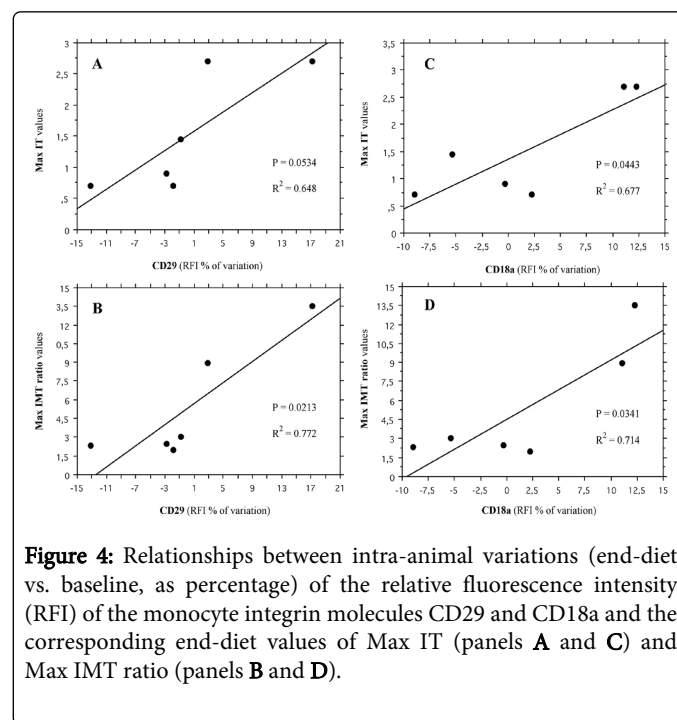
The pre-diet values of the soluble ICAM-1 showed a high inter-animal variability (CV=58%) and significantly correlated both with the end-diet values of histopathologic parameters used to quantify maximal atherosclerotic progression stage, such as max IT and max IMT ratio (Fig. 3, right panels), as well as with average atherosclerotic burden/progression parameters, such as Frequency of Atheromas (P=0.0395, R<sup>2</sup>=0.694) and Mean IT (P=0.0292, R<sup>2</sup>=0.734).

A positive correlation was observed between intra-animal variations (end-diet vs. baseline, as percentage) of the relative RFI values of the CD29 integrin subunit and the corresponding histopathologic indexes of maximal ATS progression such as Max IT and Max IMT ratio (Figure 4, panels A and B). A similar behavior has been found for the monocyte β<sub>2</sub>-integrin CD18a, with significant positive regressions between its RFI changes and the histopathologic parameters Max IT and Max IMT ratio (Figure 4, panels C and D).

For each animal, positive significant regressions were observed between ATS stage and the corresponding end-diet RFI values of CD29 (P=0.0212, R<sup>2</sup>=0.772) and CD18a (P=0.0231, R<sup>2</sup>=0.763). Similarly, a strongly positive significant relationship was observed between the end-diet CD16 RFI values and the corresponding histopathologic parameters of ATS progression, Max IT (P=0.0093, R<sup>2</sup>=0.847) and Max IMT ratio (P=0.0020, R<sup>2</sup>=0.927).

Moreover, we observed that the baseline values of the CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction (CV=19%) showed positive but not significant trends with the histopathologic parameters Mean IT (P=0.0530, R<sup>2</sup>=0.649) and Frequency of Atheromas (P=0.0823, R<sup>2</sup>=0.571). On the other hand, the individual variations (end-diet vs. baseline, as percentage) of this monocyte subset showed clearly inverse trends, even if statistically not significant, with all histopathologic parameters (data not shown). Finally, a significant positive linear regression (P=0.0100, R<sup>2</sup>=0.842) was observed between individual

HDL variations and the corresponding changes (as percentage) of the CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction.



**Figure 4:** Relationships between intra-animal variations (end-diet vs. baseline, as percentage) of the relative fluorescence intensity (RFI) of the monocyte integrin molecules CD29 and CD18a and the corresponding end-diet values of Max IT (panels A and C) and Max IMT ratio (panels B and D).

## Discussion

### Cumulative diet-induced blood monocytes phenotype changes and characterization of coronary ATS burden/progression

In the present study we employed a swine model of CAD in order to evaluate, by flow cytometry, HCD-induced phenotype modifications of circulating monocytes and their relationship with severity and extent of atherosclerotic lesions assessed by histomorphometry.

To our knowledge, this study design represents an original link between coronary histomorphometry and blood immune cell flow cytometry data in a swine model of experimentally induced CAD. In particular, once associated with post-diet histopathologic parameters quantifying either ATS extent in the entire coronary tree (Mean IT and Mean IMT ratio), or the features of most advanced localized atherosclerotic lesions (Max IT and Max IMT ratio), these data could help in clarifying the relationship between innate immune-mediated mechanisms and ATS initiation and progression [26].

Following 4 months HCD, we observed a decreased number of circulating monocytes associated with a down-modulated expression of monocyte differentiation antigen CD14 and an increased fraction of cells expressing the activation marker SLA-DR. According to previous studies demonstrating that porcine blood monocytes differentiate towards a more mature macrophage-like phenotype characterized by up-regulation of SLA-DR and down-regulation of CD14 [25], our findings can reflect a diet-induced macrophage-like maturation of circulating monocytes and their increased retention into coronary arterial sites prone to develop atherosclerotic lesions.

## Blood monocyte phenotype and endothelial markers as predictors of ATS burden/progression

The results of our study suggest the capability of some baseline (pre-diet) cellular and soluble markers to predict CAD severity and extent of lesions in the swine model of HCD-induced coronary ATS. In fact, for each animal, the pre-diet values of SLA-DR positive monocyte fraction resulted significantly correlated to end-diet coronary ATS stage and extent, independently of blood level variations of the known pro-atherogenic (oxLDL) and athero-protective (HDL) lipoproteins [27,28].

Similarly, the pre-diet levels of sICAM-1, an endothelial activation marker [29,30], significantly correlated with final average ATS burden and stage of disease, in agreement with previous clinical studies relating basal levels of sICAM-1 and ATS progression [31,32].

Moreover, pre-diet sICAM-1 levels are tightly related to monocyte positivity of the activation marker SLA-DR, which is found to correlate, in its turn, with baseline monocyte expression of CD29 integrin subunit. All above observations might support the hypothesis of a monocyte-induced endothelial dysfunction. The CD29 subunit forms, in association with the CD49d  $\alpha$ 4-integrin subunit, the very late antigen-4 (VLA-4), which represents the main integrin complex involved in monocyte adhesion to endothelial cell [33,34]. The importance of the CD29 molecule expression level (in terms of RFI) on blood monocytes in influencing ATS progression has been further confirmed for the first time in this study by the positive correlations between individual RFI variations of this integrin subunit expression and coronary ATS severity and extent. A similar behavior is found for the monocyte  $\beta$ 2-integrin CD18a that shows significant positive relationships with ATS stage.

All these findings suggest that pre-diet functional state of circulating monocytes could influence baseline endothelial activation state itself, probably as a consequence of an individually modulated surface expression of adhesion molecules and/or co-stimulatory receptors able to bind to specific endothelial ligands [35]. No previous experimental or clinical studies have proposed such a mechanism of ATS initiation.

## Pathophysiologic and clinical hypothesis

Previous human studies have demonstrated the usefulness of flow cytometry quantification of integrin molecules expression on circulating monocytes, from a single blood drawing, in predicting the progression of coronary and peripheral atherosclerotic arterial disease under different clinical conditions [30,36]. Under this aspect, the significantly positive relationships observed for the first time in our study between ATS stage and corresponding end-diet RFI values of CD29 and CD18a propose these integrin molecules as candidate markers to monitor ATS coronary lesion progression.

Baseline fractions of SLA-DR positive monocytes have been found to marginally correlate also with corresponding values of CD16 expression. The CD16 (or Fc $\gamma$ RIII) is the low-affinity receptor for the IgG Fc portion and its higher expression on swine monocyte lineages indicates an activation state and a more mature stage of differentiation towards macrophages [37]. Previous clinical studies demonstrated the importance of CD16 quantification on distinct monocyte subsets in predicting adverse cardiovascular events in subjects recruited for elective coronary angiography [38]. However, to our knowledge, neither clinical nor experimental studies evaluated, so far, the

relationships between CD16 expression level (quantified as RFI) on all circulating monocytes and coronary ATS evolution. In our study, for the first time, the end-diet CD16 RFI values derived from all blood monocytes have been shown to correlate strongly with ATS maximal severity, suggesting a relevant association between this monocyte activation marker and ATS progression. On this basis, the above mentioned pro-adhesive and activation monocyte surface antigens could represent not only useful markers to monitor ATS progression, but also potential targets for newly developed immune-based therapies.

Pig monocytes have been divided into two major subpopulations on the basis of the CD163 marker expression [24]. As in humans, the CD163 expression in pigs is restricted to monocyte/macrophages and clearly defines a distinctive monocyte subset [13,39]. In particular, the simultaneous flow cytometry analysis of CD14 and CD163 expression on pig blood monocytes showed that CD163 is expressed on the CD14<sup>low</sup> cell subset at higher level than on CD14<sup>high</sup> monocytes [13]. We quantified the CD172-derived CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction (as percentage) by using a CD14 fluorescence histogram-based median channel and a bivariate fluorescence dot-plot of CD163 vs. CD14, as already reported [13]. We found that, for each animal, the baseline fraction of CD14<sup>low</sup>/CD163<sup>high</sup> monocytes correlated significantly with the corresponding percentages of positivity of other activation markers, in particular CD16 and SLA-DR, on all blood monocytes. Moreover, the presence of a positive significant relationship between the intra-animal HDL variations and the corresponding changes of the CD14<sup>low</sup>/CD163<sup>high</sup> monocyte fraction, which are inversely related to the histological features of coronary ATS burden, suggests the hypothesis of a HDL-induced maturation of swine blood monocytes towards a CD14<sup>low</sup>/CD163<sup>high</sup> phenotype that could act in an athero-protective manner, probably as a consequence of a prevalent M2 functional polarization of this subset [40,41].

## Limits of the study

The main study limitation is the low number of studied animals, which restricts the statistical robustness of the relationship between circulating monocyte markers expression and HCD-induced histomorphometric indexes of CAD.

Another limitation is represented by the lack of quantification of macrophages and foam-cells infiltration into ATS lesions and the correlation with the expression level of circulating monocyte markers. However, preliminary histological observations evidenced a low macrophage/foam cell infiltration into coronary lesions after 4 months of HCD, probably reflecting a reduction of monocyte trafficking into ATS coronary lesions. Another study limitation is the absence of blood monocyte chemokine receptors expression evaluation (in particular of CCR2, CCR5 and CX3CR1). This limitation, actually due to the lack of a panel of commercially available monoclonal antibodies specific for the swine chemokine receptors, will represent a future challenge to better understand the role of monocyte trafficking in plaque formation and progression in this experimental model. Under a translational viewpoint, the recent evidence of a significant correlation between carotid plaque inflammation, as assessed by [18F]-fluorodeoxyglucose positron emission/computer tomography technique, and the intraplaque CD68 positive cell content [42], raises the appealing possibility of a non-invasive evaluation of monocyte contribution to ATS plaque progression/evolution in humans.

## Conclusions

Taken together, our preliminary data suggest the potential relevance of circulating monocyte phenotype features in predicting CAD stage and progression, assessed by histology, in a swine model of experimentally induced ATS. In agreement with human clinical trials, demonstrating the importance of the circulating immune effector characterization in predicting cardiovascular disease outcome [43], our experimental findings, albeit preliminary, support a role of the circulating monocyte functional state in coronary plaque initiation and early progression, providing the basis for a better knowledge of immune cell contribution to CAD development in humans.

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