Supplemental Material to Campo et al. "Biological Effects of Ticagrelor over Clopidogrel in Patients with Stable Coronary Artery Disease and Chronic Obstructive Pulmonary Disease"

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Supplemental Methods

Inclusion criteria

1. Age ≥18 years;
2. Ability to provide informed written consent and to participate in the 6-months follow-up period;
3. Diagnosis of stable coronary artery disease requiring coronary artery angiography, percutaneous coronary intervention and stent implantation
4. chronic obstructive pulmonary disease diagnosis confirmed by spirometry in stable phase and in stable medical treatment from at least 3 months.

Exclusion criteria

1. Patients hospitalized with diagnosis of acute coronary syndrome
2. Previous chronic use of $P2Y_{12}$ inhibitors
3. Known intolerance to aspirin and/or $P2Y_{12}$ inhibitors
4. Significant variation in guideline driven medical treatment in the last 15 days
5. History of intracranial haemorrhage
6. Known intake of a strong CYP3A4 inhibitor or inducer;
7. Known pregnancy, breast-feeding, or intend to become pregnant during the study period
8. Planned surgery, including coronary artery bypass graft or a staged procedure within 6 months;
9. Known moderate to severe hepatic impairment (alanine-aminotransferase $\geq$ 3 x ULN);
10. Need for chronic oral anti-coagulation therapy;
11. Active major bleeding or major surgery within the last 30 days;
12. Known stroke (any type) within the last 30 days;
13. Currently participating in another trial before reaching primary endpoint;
14. Thrombocytopenia;
15. Increased risk of bradycardia;
16. Known other inflammatory chronic disorders;
17. Known or suspected malignancy
18. Other concomitant pulmonary diseases

Endothelial function analysis

- **Apoptosis in human umbilical vein endothelial cells**

Apoptosis levels were assessed in with the Annexin V-FITC binding assay (modified from Agnoletti et al, 1999). Briefly, human umbilical vein endothelial cells (HUVECs) purchased from Invitrogen (Carlsbad, CA, USA) were grown for 48 h in the presence of 20% serum from patients then cells were collected and stained with Annexin V-FITC (Life Technologies, Carlsbad, CA, USA) (100 ng/ml) and propidium iodide (Sigma-Aldrich, Saint Louis, MO, USA) (10 mg/ml) at room temperature in the dark for 15 min. Flow cytometric analysis was performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA, USA), for each sample 20,000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA, USA).

- **Nitric Oxide (NO)**

Nitric oxide levels were assessed in HUVECs grown for 48 h in the presence of 20% serum from patients. Cells were collected and stained and washed with PBS then re-suspended in PBS containing 1uM of DAF-FM-DA (nonfluorescent 4-amino-5-methylamino-2',7' difluorofluorescein diacetate, Thermo Fisher Scientific, Waltham, MA, USA), and incubated in the presence of 10mM Arginine (Sigma-Aldrich, Saint Louis, MO, USA) at room temperature for 30 min in the dark. Cells were then washed, re-suspended in PBS, and kept on ice for an immediate detection by flow cytometry. Flow cytometric analysis was performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA, USA), for each sample 15,000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA, USA).

- **Reactive oxygen species (ROS) in peripheral blood mononuclear cell (PBMC)**

ROS levels were detected in PBMC by flow cytometry (modified from Sarkar et al, 2005). Whole blood samples were collected in sterile tubes coated with anticoagulants Vacutainer® (BD Diagnostics, Sparks, MD, USA). PBMC were isolated from whole blood using Ficoll-Paque Plus (GE Healthcare Life Sciences, Pittsburgh, PA, USA) by gradient centrifugation according to manufacturer's instructions. Isolated PBMC was washed with PBS and re-suspended. For ROS flow-cytometric analysis 10µ MDCFH-DA (Thermo Fisher Scientific, Waltham, MA, USA) was added to $1x10^6$ cells and incubated at 37°C for 30 minutes in dark. Cells were then washed, re-suspended in PBS, and kept on ice for an immediate detection by flow cytometry. Analysis was performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA, USA), for each sample 15,000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA, USA).

Inflammation parameters (cytokines/chemokines) analysis

Serum samples were stored at -80°C and thawed only once before performing the MILLIPLEX MAP Human Cytokine/Chemokine Panel (Merck Millipore, Billerica, MA) a bead-based multiplex immunoassay, which allows the simultaneous detection and quantification of the following 29 human cytokines/chemokines: epidermal growth factor (EGF), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte monocyte colony-stimulating factor (GM-CSF), interferon (IFN) -α2, IFN-γ, interleukin (IL) -10, IL-12(p40), IL-12(p70), IL-13, IL-15, 1L-17α, IL-1 receptor antagonist (ra), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, inducible protein (IP) -10 (CXCL10), monocyte chemoattractant protein (MCP) -1, macrophage inflammatory protein (MIP) -1α, MIP-1β, tumor necrosis factor (TNF) -α, TNF-β, and vascular endothelial growth factor (VEGF). Samples were processed following the manufacturer's instructions. Data were analyzed by MAGPIX instrument provided with the MILLIPLEX-Analyst Software.

Supplemental Tables

Suppl. Table 1. Citokines/chemokines in serum from patients.

	Clopidogrel (n=23)	Ticagrelor (n=23)	p
Baseline EGF, (%)	202±96	211±84	0.7
1-month EGF, (%)	175±98	148±70	0.2
N below the detection limit	0/0	0/0	0.9
Baseline Eotaxin, (%)	206±56	214±70	0.7
1-month Eotaxin, (%)	197±61	234±102	0.2
N below the detection limit	0/0	0/0	0.9
Baseline G-CSF, (%)	3.2[3.2-9.7]	3.2[3.2-5.67]	0.6
1-month G-CSF, (%)	3.2[3.2-10.7]	3.2[3.2-3.2]	0.2
Pts below the detection limit	14/14	17/19	0.6
Baseline GM-CSF, (%)	1.3[1.3-1.3]	1.3[1.3-1.8]	0.8
1-month GM-CSF, (%)	1.3[1.3-4.3]	1.3[1.3-1.3]	0.9
N below the detection limit	18/13	17/17	0.8
Baseline IFN alpha, (%)	2.7[2.7-2.7]	2.7[2.7-2.7]	0.9
1-month IFN alpha, (%)	2.7[2.7-2.7]	2.7[2.7-2.7]	0.9
N below the detection limit	20/18	20/19	0.9
Baseline IFN gamma, (%)	2.4±2.9	2.4±2.5	0.9
1-month IFN gamma, (%)	4±4	3±4	0.4
N below the detection limit	12/8	12/12	0.9
Baseline IL-10, (%)	1.5[1.5-1.5]	1.5[1.5-1.5]	0.9
1-month IL-10, (%)	1.5[1.5-3.3]	1.5[1.5-1.5]	0.9
N below the detection limit	17/15	22/21	0.9
Baseline IL-12p4, (%)	3.1[3.1-3.1]	3.1[3.1-3.1]	0.9
1-month IL-12p4, (%)	3.1[3.1-3.1]	3.1[3.1-3.1]	0.9
N below the detection limit	18/18	22/20	0.9
Baseline IL-12p7, (%)	1.6[1.6-1.6]	1.6[1.6-1.6]	0.9
1-month IL-12p7, (%)	1.6[1.6-1.6]	1.6[1.6-1.6]	0.9
N below the detection limit	20/20	20/20	0.9
Baseline IL-13 (%)	1.8[1.8-1.8]	1.8[1.8-1.8]	0.9
1-month IL-13, (%)	1.8[1.8-1.8]	1.8[1.8-1.8]	0.9
N below the detection limit	18/18	22/21	0.9
Baseline IL-15, (%)	1.1[1.1-1.8]	1.1[1.1-1.1]	0.9
1-month IL-15, (%)	1.6[1.6-2.2]	1.6[1.6-1.6]	0.9
N below the detection limit	16/16	22/21	0.9
Baseline IL-17A, (%)	1.1[1.1-1.1]	1.1[1.1-2.9]	0.8
1-month IL-17A, (%)	1.1[1.1-1.1]	1.1[1.1-3.3]	0.8
N below the detection limit	19/17	16/14	0.7
Baseline IL-1RA, (%)	18±21	16.5±20	0.8
1-month IL-1RA, (%)	18.3±24	15±16	0.6
N below the detection limit	8/8	8/6	0.8
Baseline IL-1a, (%)	4.3[4.3-4.3]	4.3[4.3-4.3]	0.9
1-month IL-1a, (%)	4.8[4.8-4.8]	4.8[4.8-4.8]	0.9
N below the detection limit	18/18	21/20	0.7

Baseline IL-1b, (%)	1.5[1.5-1.5]	1.5[1.5-1.5]	0.9
1-month IL-1b, (%)	1.5[1.5-1.5]	1.5[1.5-1.5]	0.9
N below the detection limit	21/21	22/21	0.9
Baseline IL-2, (%)	0.4[0.4-0.4]	0.4[0.4-0.4]	0.9
1-month IL-2, (%)	0.4[0.4-0.4]	0.4[0.4-0.4]	0.9
N below the detection limit	18/18	22/21	0.9
Baseline IL-3, (%)	1[1-1]	1[1-1]	0.9
1-month IL-3, (%)	1[1-1]	1[1-1]	0.9
N below the detection limit	21/21	22/21	0.9
Baseline IL-4, (%)	2.1[2.1-2.1]	2.1[2.1-2.1]	0.9
1-month IL-4, (%)	2.1[2.1-2.1]	2.1[2.1-2.1]	0.9
N below the detection limit	17/18	18/21	0.6
Baseline IL-5, (%)	0.4[0.4-0.4]	0.4[0.4-0.4]	0.9
1-month IL-5, (%)	0.4[0.4-0.4]	0.4[0.4-0.4]	0.9
N below the detection limit	19/20	15/18	0.8
Baseline IL-6, (%)	0.7[0.7-1.6]	0.7[0.7-4.6]	0.7
1-month IL-6, (%)	0.6[0.6-2]	0.6[0.6-1.3]	0.8
N below the detection limit	15/15	19/15	0.5
Baseline IL-7, (%)	0.5[0.5-0.5]	0.5[0.5-0.5]	0.9
1-month IL-7, (%)	0.5[0.5-0.5]	0.5[0.5-0.5]	0.9
N below the detection limit	21/20	19/19	0.7
Baseline IL-8, (%)	6.5[4.4-18]	4.5[6.7-20.7]	0.3
1-month IL-8, (%)	12.3[7.5-33.5]	10.6[6.9-24.9]	0.6
N below the detection limit	0/0	0/0	0.9
Baseline IP10, (%)	317.5±213	319±180	0.9
1-month IP10, (%)	346±228	299±137	0.4
N below the detection limit	0/0	0/0	0.9
Baseline MCP-1, (%)	612.3±233.9	649.5±240.8	0.6
1-month MCP-1, (%)	686.6±351.4	691.8±241.3	0.9
N below the detection limit	0/0	0/0	0.9
Baseline MIP-1a, (%)	5.6[1.5-18.8]	4.5[1.5-10.7]	0.8
1-month MIP-1a, (%)	4.3[1.5-25.1]	3.9[1.5-13]	0.6
N below the detection limit	8/10	13/10	0.6
Baseline MIP-1b, (%)	37.99±27.2	30.92±18.8	0.3
1-month MIP-1b, (%)	43.2±32	29.3±16.4	0.8
N below the detection limit	0/0	0/0	0.9
Baseline TNF alpha, (%)	10.1±5.8	11.4±5.7	0.4
1-month TNF alpha, (%)	14.7±11	12.3±8.1	0.4
N below the detection limit	0/0	0/0	0.9
Baseline TNF beta, (%)	1.2[1.2-1.2]	1.2[1.2-1.2]	0.9
1-month TNF beta, (%)	1.2[1.2-1.2]	1.2[1.2-1.2]	0.9
N below the detection limit	18/18	22/21	0.7
Baseline VEGF, (%)	175.7±175.1	178.91±177.8	0.9
1-month VEGF, (%)	198.7±179.8	201.9±213.3	0.9
N below the detection limit	0/0	0/0	0.9

p: for the comparison clopidogrel vs. ticagrelor.

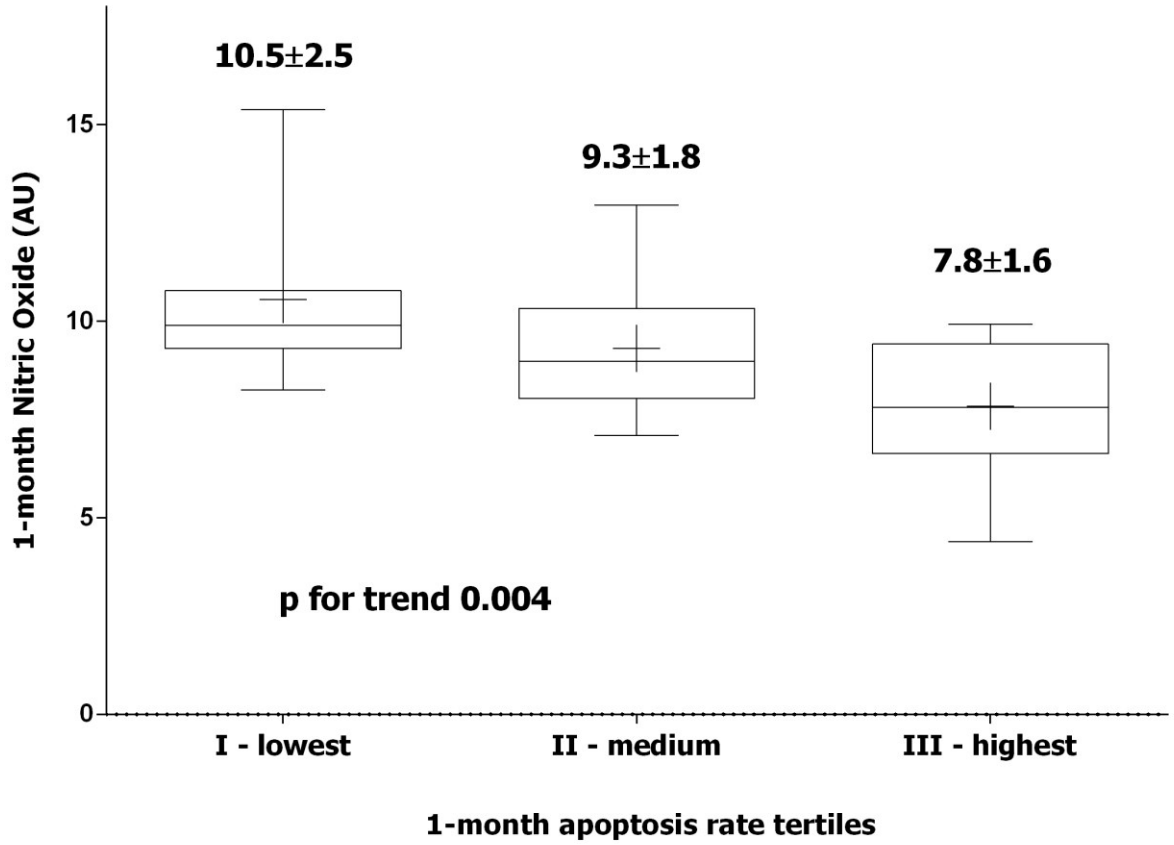
N below the detection limit: we report the number of patients with cytokine/chemokine below the detection limit for the specific assay. The first number is referred to baseline blood sample, whereas the second is referred to the 1-month blood sample. EGF: epidermal growth factor. G-CSF: granulocyte colony-stimulating factor. GM-CSF: granulocyte monocyte colony-stimulating factor. IFN: interferon. IL: interleukin. IL-1 RA: IL-1 receptor antagonist. IP-10: inducible protein 10. MCP: monocyte chemoattractant protein. MIP: macrophage inflammatory protein. TNF: tumor necrosis factor. VEGF: vascular endothelial growth factor.

Suppl. Table 2. Relation between PRU, rate of apoptosis, NO and ROS values.

	baseline rate of apoptosis		**baseline NO**		**baseline ROS**	
	r2	p	r2	p	r2	p
baseline PRU	0.7	0.1	0.002	0.7	0.003	0.6
	1-month rate of apoptosis		**1-month NO**		**1 month ROS**	
	r2	p	r2	p	r2	p
1-month PRU	0.9	0.09	0.015	0.4	0.04	0.15

NO: nitric oxide. ROS: reactive oxygen species. PRU: P2Y12 reaction unit.

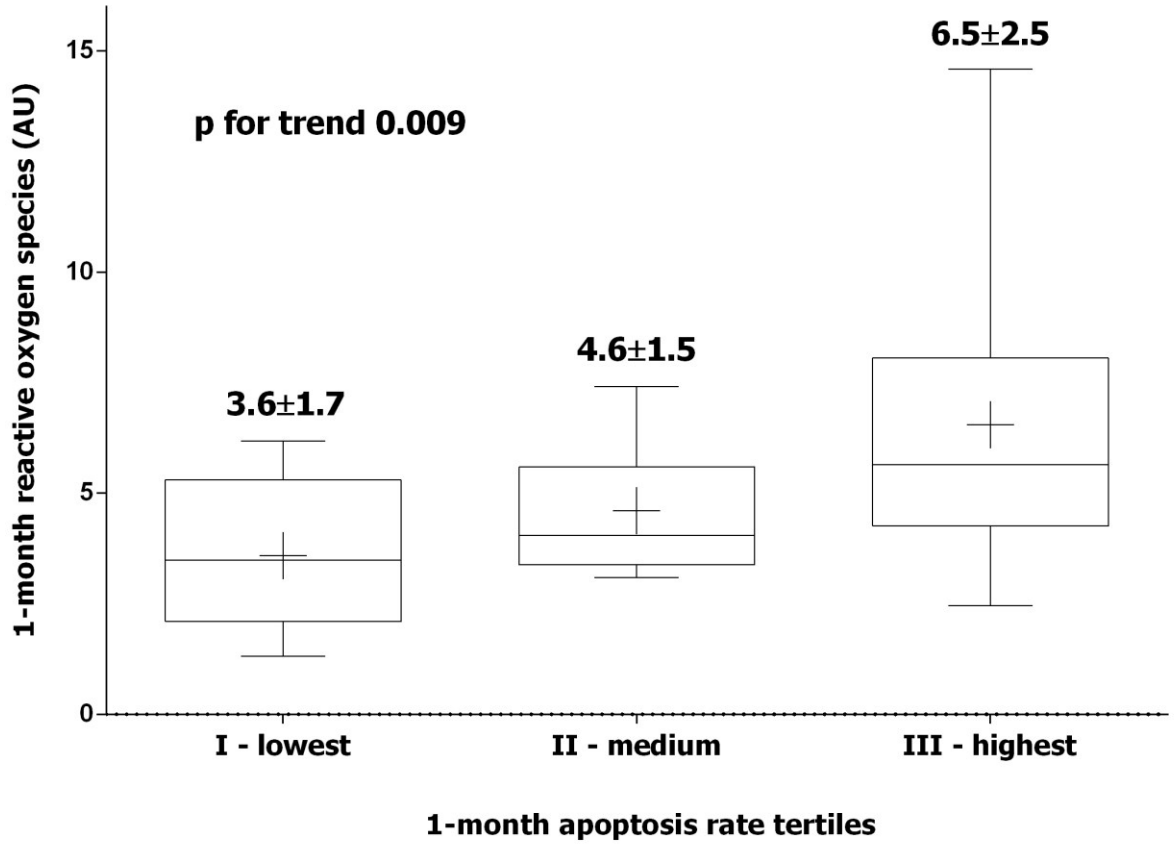
Suppl. Figure 1: 1-month NO values in patients stratified according tertiles of 1-month apoptosis rate.



NO: nitric oxide.

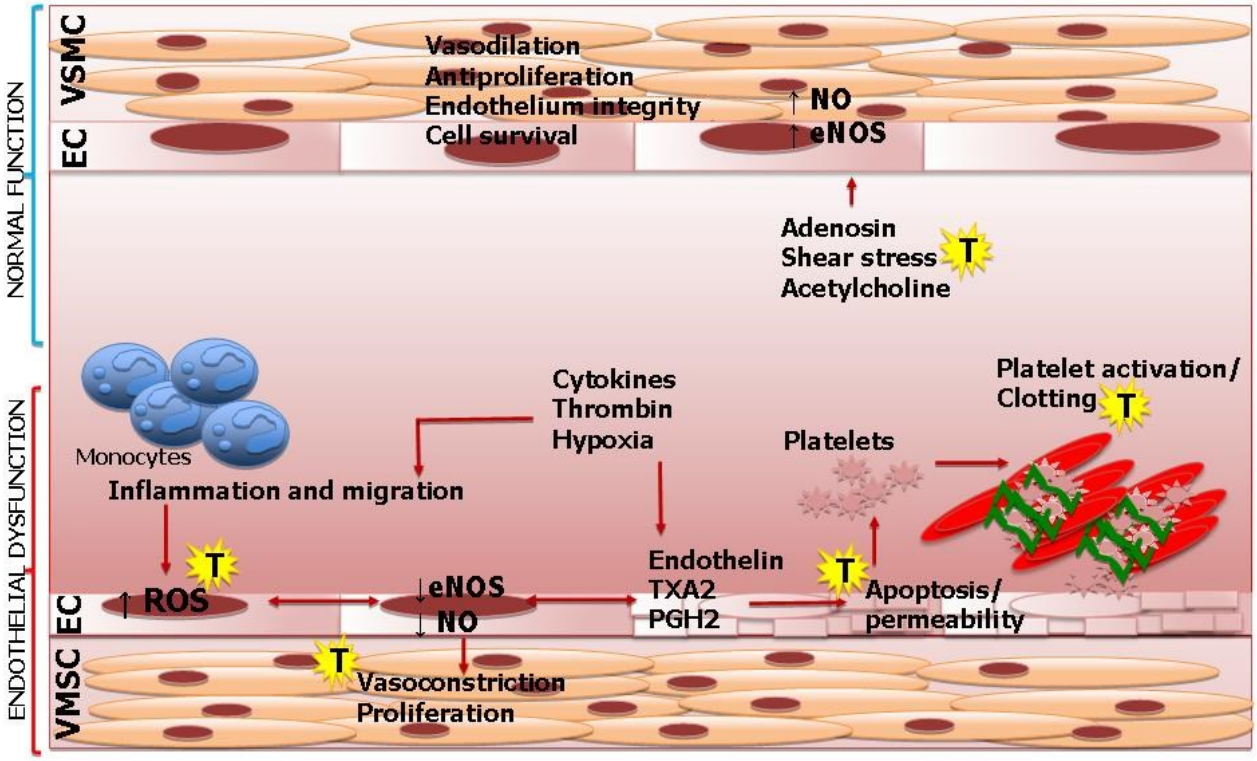
horizontal line in the box: median value. +: mean value. box: interquartile range. Vertical line: 10th to 90th range.

Suppl. Figure 2. 1-month ROS values in patients stratified according tertiles of 1-month apoptosis rate.



ROS: reactive oxygen species. horizontal line in the box: median value. +: mean value. box: interquartile range. Vertical line: 10^{th} to 90^{th} range.

Suppl. Figure 3. Main pathways involved in endothelial function regulation.



EC: endothelial cell. VSMC: vascular smooth muscle cell. NO: nitric oxide. eNOS: endothelial nitric oxide synthase. TXA2: Thromboxane A2. PGH2: Prostaglandin H2. ROS: reactive oxygen species. Several mediators are involved in the regulation of endothelial function. Adenosine is an important anti-inflammatory molecule that modulates a several processes in the vascular system. Of relevance, adenosine is able to reduce ROS in neutrophils [1-2] and to improve NO production in ECs [3]. Ticagrelor, increasing adenosine levels, may exert a beneficial effect on endothelial function (yellow sticker with T letter) [4-5].

Suppl. References

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