Microbiota Thrombus Colonization May Influence Athero-Thrombosis in Hyperglycemic Patients With ST Segment Elevation Myocardial Infarction (STEMI). MARIANELLA Study

Celestino Sardu (✉ drsarducele@gmail.com)

University of Campania <https://orcid.org/0000-0001-5099-3790>

Maria Consiglia Trotta

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Biagio Santella

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Nunzia D' Onofrio

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Michelangelo Barbieri

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Maria Rosaria Rizzo

Università degli Studi della Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Ferdinando Carlo Sasso

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Lucia Scisciola

University of Campania Vanvitelli

Fabrizio Turriziani

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Michele Torella

University Vanvitelli

Michele Portoghese

Sassari University Hospital: Azienda Ospedaliero Universitaria di Sassari

Francesco Loreni

University Vanvitelli

Simone Mureddu

Sassari University Hospital: Azienda Ospedaliero Universitaria di Sassari

Maria Antonietta Lepore

University Vanvitelli

Massimiliano Galdiero

University Vanvitelli

Gianluigi Franci

University Vanvitelli

Veronica Folliero

University Vanvitelli

Arianna Petrillo

University Vanvitelli

Lara Boatti

University Vanvitelli

Fabio Minicucci

Cardarelli Hospital: Ospedale Cardarelli

Ciro Mauro

Cardarelli

Paolo Calabrò

University Vanvitelli

Marisa De Feo

University of Campania Luigi Vanvitelli: Università degli Studi della Campania Luigi Vanvitelli

Maria Luisa Balestrieri

University Vanvitelli

Danilo Ercolini

Federico II

Michele D' Amico

Vanvitelli

Giuseppe Paolisso

University Vanvitelli

Marilena Galdiero

Vanvitelli

Raffaele Marfella

University Vanvitelli

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Abstract

Objectives. We examined the association of the coronary thrombus microbiota and relative metabolites with major adverse cardiovascular events (MACE) in hyperglycemic patients with ST segment elevation myocardial infarction (STEMI).

Background. Hyperglycemia during STEMI may affect both development and progression of coronary thrombus via gut and thrombus microbiota modifications.

Methods. We undertook an observational cohort study of 146 first STEMI patients treated with primary percutaneous coronary intervention (PPCI) and thrombus-aspiration (TA). Patients were clustered, based on admission blood glucose levels, in hyperglycemic (≥ 140 mg/dl) and normoglycemic (< 140 mg/dl). We analyzed gut and thrombus microbiota in all patients. Moreover, we assessed TMAO, CD40L and von Willebrand Factor (vWF) in coronary thrombi. Cox regressions were used for the association between *Prevotellasp* and TMAO tertiles and MACE. MACE endpoint at 1 year included death, re-infarction, unstable angina.

Results. In fecal and thrombus samples, we observed a significantly different prevalence of both *Prevotellasp* and *Alistipessp*. between patients with hyperglycemia (n=56) and those with normal glucose levels (n=90). The abundance of *Prevotella* increased in hyperglycemic vs normoglycemic patients whereas the contrary was observed for *Alistipes*. Interestingly, in coronary thrombus, the content of *Prevotella* was associated with admission blood glucose levels ($p < 0.01$), thrombus dimensions ($p < 0.01$), TMAO, CD40 ($p < 0.01$) and vWF ($p < 0.01$) coronary thrombus contents. Multivariate Cox-analysis disclosed a reduced survival in patients with high levels of *Prevotella* and TMAO in coronary thrombus as compared to patients with low levels of *Prevotella* and TMAO, after 1-year follow up.

Conclusions. Hyperglycemia during STEMI may increase coronary thrombus burden via gut and thrombus microbiota dysbiosis characterized by an increase of *Prevotella* and TMAO content in thrombi.

Background

Glycemic and coagulative disorders may play a role in both the development and progression of coronary thrombus in patients with acute myocardial infarction (AMI). However, the mechanisms are not completely understood (1). The potential role of admission hyperglycemia in modulating the gut microbiota effects in coronary thrombus development remains unclear, and only small studies with diverse methods and findings have been reported so far (2–4). Common microbiota changes observed in obesity, diabetes mellitus and dyslipidemia include a decrease in the ratio of bacteria, such as Gram-positive Firmicutes to Gram-negative Bacteroidetes, as well as either an overabundance or depletion of certain species, including *Prevotella* (5–8). The consequent effects of these shifts include alterations in the metabolic composition of the gut and increased levels of trimethylamine N-oxide (TMAO). TMAO, an amine oxide, is synthesized from choline, betaine and carnitine, which are transformed to trimethylamine (TMA) by human gut microbiota (8, 9). TMA is then oxidized to TMAO by the hepatic flavin

monooxygenase 3 (FMO3) (10). Among the genera of gut microbiota, Prevotella has showed the strongest association with TMAO production (11). Baseline measures of 53 participants in a controlled feeding study showed that those with a Prevotella enterotype had higher plasma TMAO concentrations than those with a Bacteroides enterotype (12). Moreover, recent data showed the involvement of both Prevotella (14) and TMAO (13) in the arterial thrombosis. To date, evidence about the possible interaction between hyperglycemia, microbiota and its metabolites in the development and progression of coronary thrombus in ST-elevation myocardial infarction (STEMI) patients is lacking. To define a more robust evidence of gut microbiota signature and TMAO in coronary thrombus development and progression during AMI, we assessed both gut and thrombus microbiota in hyperglycemic (blood glucose levels >140 mg/dl at admission) and normoglycemic patients with STEMI. Furthermore, we explored associations between thrombus microbiota and TMAO levels, and composite endpoints (MACE= death, re-infarction, unstable angina) in cohorts at 1-year.

Materials And Methods

Patients and study design

This was a multicenter observational prospective cohort study aimed to investigate the relationship between gut microbiota signature, TMAO thrombus levels, and 1-year outcomes in STEMI patients. We examined an observational cohort of consecutive patients with first STEMI treated with PPCI and thrombus aspiration (TA), between February 2016 and September 2019 at the Department of Cardiology of Cardarelli Hospital in Naples Italy, at Department of Cardiology of the University of Campania “Luigi Vanvitelli” Italy, and at Department of Cardiac Surgery of the SS. Annunziata Hospital, Sassari, Italy. All patients with onset of symptoms <12 h and at least 1-mm ST-segment elevation in 2 or more contiguous limb leads or at least 2 mm in 2 or more contiguous precordial leads or left bundle branch block were considered eligible for PPCI. Coronary angiography was performed either via the radial or femoral artery. The culprit lesion was identified and crossed with an angioplasty guidewire. Manual TA was performed at the discretion of the operator, due to technical aspects (e.g., type and number of stents, use of any other devices), and with consideration of angiographic selection criteria (e.g., the presence of a visible thrombus on angiography), followed by conventional PCI to the culprit vessel. We considered eligible for the study all patients with: correspondence between ECG findings and suspected culprit artery; a minimum visual estimate of 50% stenosis in the culprit artery, and feasibility of performing TA, as judged by the treating physician; age of 18 years or greater; presentation to the cardiac catheterization laboratory for PPCI in the setting of first STEMI. Patients with left ventricular ejection fraction less than 25%, with previous myocardial infarction or previous PPCI and/or coronary by-pass grafting, or who had received fibrinolytic therapy were instead excluded from the study.

Based on the admission blood glucose levels, patients were clustered in two groups: (a) hyperglycemic STEMI patients (glucose levels ≥ 140 mg/dl), as suggested by the position statement of the American Heart Association (AHA) (15) and normoglycemic STEMI patients (glucose levels <140 mg/dl). The study is in accordance with the principles outlined in the 1976 the Declaration of Helsinki and its later

amendments for use of human tissue or subjects. The Institutional Review Board of University of Campania “Luigi Vanvitelli” Italy approved the protocol.

Procedures

Routine analyses were obtained on admission before coronary angiography and before the initiation of full medical therapy. Stool samples were instead obtained during the first day after admission. Treatment was classified as “thrombus aspiration” when TA was attempted as suggested by the operator, irrespective of procedure success. Thrombus grade was classified in accordance with Sianos et al. (16) into: Grade 0 (G0), no angiographic characteristics of thrombus present; Grade 1 (G1), possible thrombus present, with the following angiography characteristics: reduced contrast density, haziness, irregular lesion contour, or a smooth convex meniscus at the site of total occlusion suggestive, but not diagnostic, of thrombus; Grade 2 (G2), definite thrombus with largest dimension $\leq \frac{1}{2}$ the vessel diameter; Grade 3 (G3), definite thrombus, with largest linear dimension $> \frac{1}{2}$ but < 2 times vessel diameter; Grade 4 (G4), definite thrombus, with the largest dimension ≥ 2 vessel diameters; Grade 5 (G5), total occlusion, unable to assess thrombus burden due to total vessel occlusion. Detailed recommendations about TA procedure were further provided. The procedure was done by interventional cardiologists’ investigators. All patients were treated with adenosine (given 120 mcg as a fast bolus followed by 2 mg in 2 minutes) and with bolus infusion of Abciximab (0.25 mg/kg IV bolus). Patients undergoing TA received the same treatment also after thrombus aspiration. Aspiration had to be started before crossing the lesion, with a minimum of two syringes (40 mL) of aspirate recommended. Investigators were appropriately trained to ensure that the guide catheter was engaged with the coronary ostia when removing the thrombectomy catheter. Finally, the guide catheter was aspirated after thrombectomy to avoid embolization of either air or thrombus from the guide catheter.

Stool sample collection and fecal microbiome analysis

Stool sample was collected in a clean container and immediately stored at - 20 °C and processed within 48 hours. The -20°C stool samples were defrosted. 220 mg of dried stool were suspended in 1,4 ml transport buffer by vortexing and transferred to a sterile DNase/RNase-free 2.0 mL tube for enzymatic lysis. Total DNA was extracted from stool sample using a DNA isolation kit (QIAamp® DNA Stool Handbook kit, cat. no. 51504; Qiagen®, Germany), according to the manufacturer's protocols. The total genomic DNA quality was assessed by 1,8 % agarose gel electrophoresis, and the DNA concentration measured using the Qubit fluorometer with the dsDNA HS (high sensitivity) Assay Kit (Invitro-gen Q32854) to evaluate DNA yield (ng) and normalized to 1 ng/ μ L.

Illumina MiSeq sequencing of 16S rRNA gene amplicons. The V3-V4-V6 hypervariable regions of the 16S rRNA gene were amplified using the Arrow Microbiota Solution B kit (cod. AD-002.024). Amplification was performed in 20- μ L reactions with amp mix for “PCR target” and 5 μ L of DNA extract. The reactions were performed using a SimpliAmp Thermal Cycler (Applied Biosystems®, #A24812) under the following thermal profile: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and one cycle of 72 °C for 5 min and a 4 °C hold. PCR products were examined by 1,6 % agarose gel

electrophoresis and then purified using AMPure Beads XP (cat. n. A63880, Beckman Coulter, Inc.). The amplicons quality was assessed by 1,6 % agarose gel electrophoresis, product visible as a band with a molecular weight of approximately 840 bp. A 6-bp barcode sequence was added to the ends of both forward and reverse primers in the PCR “index”. PCR “index” products were examined and then purified as described above. Amplicons targets were normalized to 10nM and used to prepare the library for sequencing. The library was processed using kit MiSeq Reagent Nano Kit v2 (500-cycles) (cat. n: MS-103-1003, Illumina Inc.), following the user guidelines for the system MiSeq Illumina (“Denature and Dilute Libraries Guide”-MiSeq System). The library was corrected by adding 5% of Phix Control v3 (cat. n. FC-110-3001, Illumina Inc.) and sequenced using an Illumina MiSeq platform. The amplicons were pooled, and sequencing was conducted at Ames Center (Naples, Italy), with an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA). Obtained data were processed with the MicrobAT (Microbiota Analysis Tool) system of the SmartSeq S.r.l.

16S rRNA gene sequence analysis. Sequence analysis of low quality with a lower quality score of 20, a length less than 50 bp, or any misalignments to the primer or barcode containing chimeras were removed.

Thrombus analysis

Thrombi obtained from matched STEMI patients were analyzed for microbiota colonization, TMAO levels and pro-thrombotic molecules. All analyses were performed by 3 independent pathologists, blinded to the patients' characteristics. After TA, the specimens were frozen in liquid nitrogen, stored at - 20 °C and processed within 48 hours. After defrosted, the specimens were cut perpendicular to the long axis into two halves. The first half was used for the following ELISA analysis: CD40L (Soluble- Human ELISA Kit, Invitrogen); von Willebrand Factor(VWF) (Human von Willebrand Factor ELISA Kit - ab223864); TMAO (TMAO elisa kit: Human Trimethylamine-N-oxide Reductase ELISA Kit MBS7254766, MyBiosource). A portion of the other half of the specimen was instead analyzed for microbiota colonization as previously described for stool samples.

In vitro study of bacterial growth.

In order to analyze the growth response of *Prevotella* and *Bacteroides* toward different concentrations of glucose, bacterial strains were grown as previously described (17). Briefly, *Prevotella* and *Bacteroides* from their glycerol stocks were firstly cultured on blood agar plates, then single colonies were picked individually and cultured in an anaerobic chamber at 37°C overnight in Gifu Anaerobic Medium (OD₆₀₀ 1.0–1.5). Bacterial cultures were centrifuged at 1300 × *g* for 5 min and culture pellets were washed. Twenty microliters of the culture were then added to 2 ml medium supplemented with different concentrations of D-glucose, 7 mM, 14mM, 22 mM and 32mM. Measures of optical density at 600 nm wavelength at 0, 12h, 24h, 36h, 48h, 60h and 72 h were performed in order to assess bacterial growth rates. All experiments were performed in triplicate. One-way analysis of variance with Tukey's test was performed using GraphPad Prism (version 6 for Windows, GraphPad Software, La Jolla, CA, USA).

Blood glucose control in emergency wards.

After coronary angiography procedures, all hyperglycemic patients with blood glucose > 180 mg/dl were treated with intensive glucose control to keep blood glucose levels between 140 and 180 mg/dl, as previously described (18). Continuous insulin infusion of 50 IU Actrapid HM (Novo-Nordisk) in 50 ml NaCl (0.9% using a Perfusor-FM-pump) was started only when blood glucose levels exceeded 180 mg/dl, and adjusted to keep blood glucose between 140 and 180 mg/dl. When blood glucose fell <140 mg/dl, insulin infusion was tapered and eventually stopped. After the start of insulin infusion protocol, a glycemic control was provided every hour in order to obtain three consecutive values that were within the goal range. The infusion lasted until achievement of stable glycemic goal (140-180 mg/dl) for at least 24 h. Once the glycemic goal was preserved for 24 h, the infusion was stopped, and subcutaneous insulin was initiated. Insulin was given as short-acting insulin before meals and long-acting insulin in the evening throughout the whole hospitalization period. As for the full medical therapy, the protocol stated that the use of concomitant treatment should be as uniform as possible and in accordance with evidence-based international guidelines for STEMI (19).

Follow-up

After discharge, all patients were managed and followed quarterly for 12 months after event, as outpatients, to perform clinical evaluation, routine analyses and cardiovascular evaluation (ECG, exercise ECG, echocardiography), alongside with the objective to keep HbA1c levels <7% in diabetic patients.

Outcomes

The outcome: clinical outcomes at 1-year follow-up were major adverse cardiovascular events (MACEs). MACEs are defined as a composite of cardiovascular death, non-fatal acute coronary syndrome and heart failure.

Statistical analyses

After appropriate assessment of their normal distribution by the Shapiro-Wilk test, differences between continuous variables were analyzed either with the parametric Student t-test or the non-parametric Mann-Whitney U test. Differences between categorical variables were instead evaluated by the Chi-square (χ^2) test. P values <0.05 were considered statistically significant. The study population was equally distributed for age ($p = 0.807$). To evaluate the independent contribution of hyperglycaemia in thrombus *Prevotella* and TMAO levels, we performed a correlation between *Prevotella* and TMAO levels in coronary thrombus and blood glucose level during STEMI. Risk adjusted Cox-regression analysis curves showing survival from severe disease through days of hospitalization. Cox models were adjusted for; age, diabetes, hypertension, diabetes, new diabetes, dyslipidemia, cigarette smoking, BMI, systolic blood pressure, total cholesterol, HDL and LDL-cholesterol levels, triglycerides, TIMI scores, lesions of RCA and LM, dual antiplatelet therapy and ejection fraction. To investigate the effects of *Prevotella* and TMAO thrombi expressions on cardiovascular endpoints, we evaluated STEMI outcomes at 1-year follow-up

stratified by *Prevotella* and TMAO tertiles. Statistical analysis was performed by SPSS 23 computer program.

Results

Patient characteristics. 678 patients with first STEMI (106 with hyperglycemia) were admitted at cardiologic study centers between January 2016 to July 2019. Of these, 38 patients were excluded from the study, 26 due to an onset of symptoms longer than 12 h and 12 as they died before angiographic study. Consequently, 640 were considered eligible STEMI patients and underwent to the angiographic study (91 with hyperglycemia). After coronary study, 101 patients were excluded due to CABG indication, 11 for mental disorders, 21 for absence of coronary lesions, 99 for incomplete data, whilst 355 were not treated with TA. Thus, 152 patients treated with PPCI and TA were definitely enrolled in the study, of which 59 with hyperglycemia (38.8%) (**Supplementary figure 1**). During the follow-up, 3 normoglycemic patients and 3 hyperglycemic patients voluntarily withdraw from the study and/or were excluded due to incomplete data. Therefore, the final study cohort included 90 normoglycemic and 56 hyperglycemic patients followed for 1 year. Hyperglycemic patients had higher plasma cholesterol, low-density lipoprotein (LDL), lower high-density lipoprotein (HDL) levels and thrombus grade (**Table 1**). No differences in the troponin levels, in the median time between symptom onset and the start of angiography procedure at hemodynamic unit, in the number of coronary vessel diseases and in the number of patients with post-procedural TIMI 3 were observed (**Table 1**).

In-hospital treatments and glucose control. During hospitalization, hyperglycemic patients were treated according to the most recent international guidelines (**23**), with high proportions of patients receiving platelet inhibitors and antithrombotic agents before, during and after the procedure. In particular, aspirin was administered to 98% of patients and statins to 86% in both groups. Beta-blockers were given to 81% of patients, of whom the 43% received an ACE-inhibitor in both groups. Among the whole study population, 88% of patients were treated with a combination therapy of thienopyridine and aspirin. After PPCI, the glycemic goal was preserved for 24 h (mean glycemia 160 ± 23 mg/dl). In the 10% of patients, blood glucose <70 mg/dl both with and without symptoms occurred during insulin infusion. At hospital discharge, both fasting and post-prandial plasma glucose levels were: fasting, 133 ± 31 mg/dl, post-prandial 177 ± 19 mg/dl. In addition, the rate of ACE-inhibitors, aspirin, beta-blockers, statins, and thienopyridines use increased as compared to that on admission both in normoglycemic and hyperglycemic patients (**Table 1**).

Fecal microbiota

Differences between gut microbiota of hyperglycemic and normoglycemic STEMI patients were observed (**Figure 1**). Some bacterial taxa in the intestinal microbiota were found significantly more abundant in patients with hyperglycaemia as compared to normoglycemic patients. At the Phylum level, the Bacteroidetes are more abundant in hyperglycemic compared to normoglycemic patients (46.5% vs. 21.5%; $P = 0.007$) (**Supplementary figure 2**). However, the Firmicutes and the Actinobacteria were reduced

in hyperglycemic compared to normoglycemic patients (Firmicutes: 37.1% vs 62.1%: $P=0.003$) (Actinobacteria: 3.4% vs 10 %, $P=0.001$). At the genus level, the enriched proportions belong to the genus *Prevotella* increased by 3-fold while the *Bacteroides* were reduced by 10-fold in hyperglycemic compared to normoglycemic patients (**Supplementary figure 2**). Examination of the percentage of specific genera and bacterial species present in stool revealed that only few genera differed between hyperglycemic and normoglycemic patients (**Supplementary figure 2**). Among *Prevotella* genus, *Prevotella* DJF RP53 was almost 5-fold higher in hyperglycemic as compared to normoglycemic patients (**Supplementary figure 2**).

Within the Bacteroidetes, *Alistipes* was less represented in fecal sample from hyperglycemic patients, conversely to the well depiction in normoglycemic patients (0.51% vs 4.2%, $P<0.05$). Finally, fecal *Prevotella* content was significantly associated with admission blood glucose levels ($p<0.01$) (**Supplementary figure 2**).

Thrombus microbiota

Differences between thrombus microbiota of hyperglycemic and normoglycemic STEMI patients are reported ($p < 0.001$) (**Figure 2**). Bacteroidetes and Proteobacteria are more abundant in the hyperglycemic rather than normoglycemic patients (Bacteroidetes: 44.6% vs 19.5%, $P=0.011$ and Proteobacteria: 22.6% vs 7.6%, $P=0.001$). On the contrary, Firmicutes and Actinobacteria show a reduced prevalence among hyperglycemic as compared to normoglycemic patients (Firmicutes: 26.1% vs 60.9%, $P=0.005$ and Actinobacteria: 9.9% vs 33.5%, $P=0.001$) (**Figure 2**). At the genus level, the highest rates belong to the genus *Prevotella*, with a 5-fold increase, while *Bacteroides* disclosed a 7-fold reduction in hyperglycemic as compared to normoglycemic patients (**Figure 2**). Examination of the prevalence of specific genera and bacterial species present in thrombus revealed that few genera can be significantly different between hyperglycemic and normoglycemic patients (**Figure 2**). *Alistipes* was absent in hyperglycemic thrombi, whilst it occurred in normoglycemic patients (7.3%). Finally, thrombus *Prevotella* content was significantly associated with admission blood glucose levels ($P=0.001$) (**Figure 2**).

In-vitro study, effects of high glucose concentration on growth curve of *Prevotella* DJF RP53 and *Alistipes*. Due to the major modification of *Prevotella* DJF RP53 and *Alistipes* in hyperglycemic as compared to normoglycemic thrombi, we assessed the effects of high-glucose concentration on *Prevotella* DJF RP53 and *Alistipes* growth curve on soil. Results clearly indicated that *Prevotella* DJF RP53 growth curve was accelerated and increased in soil containing high-glucose concentration, as compared with normal glucose concentration ($P=0.012$). On the other hand, high-glucose concentration slowed and decreased the *Alistipes* growth curve on soil, as compared normal glucose concentration on soil ($P=0.016$) (**Figure 3**).

Coronary thrombus analysis in hyperglycemic and normoglycemic patients

The volume and size of the thrombi, and the number of retrieved fragments were significantly higher in hyperglycemic as compared to normoglycemic patients ($P<0.001$) (**Supplementary figure 3**). Thrombi composition displayed higher CD40L, VWF and TMAO levels as compared to normoglycemic patients

(**Supplementary figure 2**). Moreover, microbiota genus analysis revealed a marked presence of *Prevotella* in hyperglycemic thrombi which, instead, was almost absent in normoglycemic thrombi (**Supplementary figure 2**). Moreover, *Prevotella* thrombus levels were significantly associated with the size of thrombi, and TMAO, CD40L and VWF levels during STEMI (**Figure 4**).

Treatments at 12 months following discharge

Between presentation of STEMI and the 12 months of follow-up after hospital discharge, 16 patients (10.9%) in the entire study cohort disclosed a new onset of diabetes, of which 15 (26.8%) among hyperglycemic patients. Diabetic patients did not show any significant difference in metabolic control between groups in overall population (**Table 1**). Over the 1-year follow-up period, there was no difference in both anti-diabetic and cardiovascular therapies between the two groups. During 1-year follow up, there are no differences in glycemic control among the groups (data not shown)

Post-discharge outcomes. After 12 months following PPCI, a multivariable Cox regression analysis, adjusted for age, diabetes, hypertension, diabetes, new diabetes, dyslipidemia, cigarette smoking, BMI, systolic blood pressure, total cholesterol, HDL and LDL-cholesterol levels, triglycerides, TIMI scores, lesions of RCA and LM, dual antiplatelet therapy and ejection fraction, showed a significantly higher one-year overall survival for MACE in normoglycemic patients (**Figure 5**). STEMI outcomes at 1-year follow-up stratified by *Prevotella* and TMAO level tertiles in coronary thrombi of hyperglycemic and normoglycemic patients were further assessed to translate molecular results in clinical endpoints. Cox multivariable regression analysis reported a lower survival from events among patients with higher *Prevotella* and TMAO levels (**Figure 5**). Interestingly, we observed an increased risk association with *Prevotella* and TMAO tertiles, consistent regardless of the dual antiplatelet therapy during the follow-up, thus suggesting a reduction of effectiveness of anti-platelet therapy in patients with high levels of *Prevotella* and TMAO thrombus levels.

Discussion

In the present study we investigated for the first time the relationship between thrombus *Prevotella* colonization and recurrent acute coronary syndrome and death among hyperglycemic patients with first STEMI. Moreover, we analyzed the relationship between thrombus microbe colonization and thrombus size, TMAO content, as well as thrombus levels of CD40L and VWF. We observed significant differences between gut and thrombus microbiota of hyperglycemic and normoglycemic STEMI patients. Fecal and thrombus *Prevotella* contents were more abundant in hyperglycemic than normoglycemic patients and, interestingly, were also significantly associated with admission blood glucose levels. On the contrary, *Alistipes* contents were lower in fecal samples and absent in thrombus samples from hyperglycemic patients, while well represented in normoglycemic patients.

These findings raise two important considerations. First, hyperglycemia on admission, in the setting of acute coronary syndrome, may suggest a substantial change in both gut and coronary thrombus microbiota compositions. In fact, fecal and thrombus samples from hyperglycemic patients showed

higher contents of *Prevotella* and low *Alistipes* contents as compared to samples from normoglycemic patients. This hypothesis is supported by data from our *in vitro* study. Indeed, the high-glucose concentration, mimicking the hyperglycemic milieu, accelerated and increased *Prevotella* DJF RP53 growth curve and slowed and decreased *Alistipes* growth curve on soil. In addition, during acute coronary syndrome, hyperglycemia may favor thrombus colonization, thus driving intestinal barrier permeability (20). In this setting, hyperglycemia and GLUT2-dependent transcriptional reprogramming alter both tight and adherence junction integrity of intestinal epithelial cells (20). Consequently, hyperglycemia-mediated barrier disruption leads to the systemic influx of microbioma and an enhanced systemic dissemination. Moreover, treatment of hyperglycemia, intestinal epithelial-specific GLUT2 deletion, or inhibition of glucose metabolism restore barrier function and bacterial containment (20). Thus, our data suggest that the increase in the intestinal barrier permeability among patients with hyperglycemia may favor thrombus contamination in a coronary syndrome setting.

The role of both *Prevotella* and *Alistipes* in the metabolic and cardiovascular diseases has been already reported by previous studies (21). Fecal and gingival *Prevotella* increased both in diabetic and hyperglycemic patients as compared to healthy subjects (22, 23). These studies not only demonstrated a positive association of *Prevotella* gingival content with higher blood glucose levels, though also a significant contribution of *Prevotella* gingival content to the development of disease condition, via increased TMAO production (23). Accordingly, data from recent studies (11) suggest that *Prevotella*, among the microbial genera, showed the strongest association with TMAO levels. On the contrary, abundance of stool *Alistipes* has been associated with low levels of TMAO (24). There is considerable evidence linking the metaorganismal TMAO pathway to the development of atherosclerosis and thrombosis in animal models and incident cardiovascular disease risks. In particular, the proatherogenic effects of microbe-dependent TMAO formation include enhanced macrophage cholesterol accumulation and subsequent foam cell formation, proinflammatory changes in the artery wall, and promotion of both platelet hyperactivity and enhanced arterial thrombosis potential. (25). Thus, our data suggest a potential antagonistic effect between *Alistipes* and *Prevotella*, as shown by the absence of *Alistipes* and higher contents of *Prevotella* in hyperglycemic coronary thrombus, in the setting of acute coronary syndrome. This trend has been commonly seen in other heart conditions, such as atrial fibrillation and heart dysfunction (26,27). Moreover, to date, there has been no evidence about the possible role of *Prevotella* thrombus colonization in outcomes of high-risk patients (e.g., those found in hyperglycemic patients during STEMI), and about the specific pathways transducing thrombus *Prevotella* stimuli in the modulation of coronary thrombus burden.

The novelty of our study is represented by the evidence that *Prevotella* coronary thrombus content is remarkably increased and it is associated with high thrombus burden, TMAO, CDL40, and vWF in hyperglycemic patients as compared with normoglycemic patients with STEMI. Interestingly, *Prevotella* coronary thrombus contents were associated with admission blood glucose levels. These data suggest that high thrombus burden observed in hyperglycemic patients could represent a result of the *Prevotella* effects on increased TMAO levels and on the consequent effects on CDL40 and vWF thrombus contents, as reported in experimental studies that clearly underlined the role of TMAO in increasing coagulation

(13). In addition, several studies (28) suggest that fecal over-abundance of *Prevotella* is associated with an increase in insulin-resistance. The role of *Prevotella* in the human microbiome is controversial, and the relative abundance of *Prevotella* in general and *P. copri* is highly dependent on diet and lifestyle (29-31). We can speculate that hyperglycemia could activate the following harmful vicious circle: I) hyperglycemia promotes systemic diffusion of *Prevotella*; II) *Prevotella* overabundance associates with increased TMAO levels; III) high TMAO levels increase systemic IR; IV) increased insulin-resistance in turn increases hyperglycemia (Figure 6). This hypothesis may have a clinical implication since patients with higher *Prevotella* and TMAO thrombus contents were more likely to experience subsequent cardiovascular events and death. The associations observed between *Prevotella* and TMAO thrombus contents and cardiovascular events and death remained significant also after adjustment for potentially confounding clinical factors. Consistently with such evidence, in a contemporary sample of patients with STEMI treated with PPCI and TA in routine practice, we observed a higher cumulative incidence of 1-year mortality and adverse cardiovascular outcomes, in patients with higher thrombus *Prevotella* and TMAO contents. In fact, the 1-year prognosis of patients with STEMI and III tertiles of *Prevotella* (<2.72%) and TMAO (>60 ng/ml) was significantly worse, with a 12.1% rate of death or re-admission for cardiovascular diseases, as compared with prognosis of patients with STEMI and I tertiles of *Prevotella* (>1.25%) and TMAO (>36 ng/ml), which disclosed a 1.9% rate of death or re-admission due to cardiovascular diseases at follow-up. TMAO levels are also affected by diet in a microbiome-driven manner, and lower levels were associated with consumption of plant-based diets (32) although an anticipated reduction of TMAO levels was not observed upon intervention with high-vegetables Mediterranean diet in obese subjects (33). Interestingly, the 1-year follow-up results based on *Prevotella* and TMAO thrombus contents did not change despite a similar severity of atherosclerotic disease at baseline and a similar glycemic control both at discharge and at follow-up. These observations suggest that on admission hyperglycemia may play a pivotal role in the increase of coronary thrombus burden, during STEMI, favoring thrombus colonization and proliferation by *Prevotella*.

However, our study has some limitations. First, the observational prospective study design and the relatively small sample size. In addition, no power calculation could be performed, due to the lack of previous studies in this setting. As a consequence, the enrollment of patients in each group was arbitrary.

Conclusion

In conclusion, our data suggest that the coronary thrombus environment is more likely susceptible to a microbial colonization which might stimulate a pro-coagulatory response in acute coronary syndrome. These findings add new elements to the pathogenesis of ACS and suggest novel therapeutic targets.

Abbreviations

microbiota; TMAO.

Abbreviations:

HDL= high-density lipoprotein;

LDL= low-density lipoprotein;

MACE= major adverse cardiovascular events;

PPCI= primary percutaneous coronary intervention;

RCA= right coronary artery;

STEMI= ST-segment elevation myocardial infarction;

TA= thrombus aspiration;

TMAO= Trimethylamine-N-oxide.

vWF= von Willebrand Factor

Declarations

Ethics approval and consent to participate: Ethical Committees of all participating Institutions gave full consent to participate in the study. Ethical Committee number 268/2016.

Consent for publication: all authors gave full consent for study publication.

Availability of data and materials: data and materials are fully available.

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Authors' contributions: C.S, M.C.T, R.M, G.P and M.G: study writing, editing and data interpretation; B.S, N.DO, M.B, M.R.R, F.C.S, M.DA, M.G: data analysis and interpretation; L.S, F.T: data collection; M.T, M.P, F.L, S.M, M.A.L, A.B, A.R, D.DA, F.M, C.M, P.C, M.DF, D.E: performed interventions and samples collection.

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Tables

Table 1. Baseline clinical characteristics, angiographic and procedural data in overall study-population.

	Hyperglycemic patients	Normoglycemic patients	P
N	56	90	
Mean age (years)	64.1±6.8	65.5±6.5	0.204
BMI (kg/m ²)	29.2±1.9	29.7±1.9	0.183
SBP (mmHg)	119.9±8.1	120.2±7.1	0.809
DBP (mmHg)	79.2±6.9	79.3±6.4	0.927
Heart rate (bpm)	87.6±9.1	86.7±7.8	0.515
LVEF			
>50%, n (%)	46 (82.1)	69 (76.7)	0.284
41% to 50%, n (%)	8 (14.3)	17 (18.9)	0.315
25% to 40%, n (%)	2 (3.6)	4 (4.4)	0.579
Laboratory analyses			
Plasma glucose (mg/dl)	202.4±27.3	102.1±22.1	0.006
Cholesterol (mg/dl)	217.2±19.7	205.5±23.1	0.003
LDL-cholesterol (mg/dl)	143.3±19.0	132.2±22.8	0.004
HDL-cholesterol (mg/dl)	36.6±3.6	47.2±3.4	0.001
Triglycerides (mg/dl)	196.8±23.1	180.6±20.1	0.001
Creatinine (mg/dl)	1.0±.2	1.0±0.2	0.510
cTnT (ng/l)	7.9±3.0	7.6±1.2	0.353
Risk Factors			
Diabetes, n (%)	17 (30.4)	18 (20)	0.111
Hypertension , n (%)	35 (62.5)	56 (62.2)	0.558
Dyslipidemia, n (%)	16 (28.6)	29 (32.2)	0.392
Cigarette smoking, n (%)	3 (5.4)	8 (8.9)	0.366
New diabetes, n (%)	15 (26.8%)	16 (10.9%)	0.036
Active treatments			
β-blockers, n (%)	28 (50.0)	32 (35.6)	0.061
ACE inhibitors, n (%)	25 (44.6)	31 (34.4)	0.156
Angiotensin receptor blockers, n (%)	165 (16.5)	372 (37.2)	0.145

Calcium inhibitor, n (%)	6 (10.7)	11 (12.2)	0.502
Statins, n (%)	15 (26.8)	25 (27.8)	0.527
Diuretic, n (%)	7 (12.5)	11 (12.2)	0.577
Insulin, n (%)	15 (7.8)	174 (17.4)	0.449
Oral anti-diabetic drugs, n (%)	16 (28.6)	25 (27.8)	0.667
Aspirin, n (%)	50 (89.3)	84 (93.3)	0.285
Thienopyridine, n (%)	5 (8.9)	5 (5.6)	0.321
Procedural data			
Symptom to angiography, hours	6.1±1.4	6.0±1.1	0.899
Angiographic data			
<i>Number of diseased vessels</i>			
1-Vessel disease, n (%)	35 (62.5)	55 (61.1)	0.504
2-Vessel disease, n (%)	15 (26.8)	30 (33.3)	0.259
3-Vessel disease, n (%)	5 (8.9)	5 (5.6)	0.321
<i>TIMI Flow Grade</i>			
Grade 0, n (%)	13 (23.2)	25 (27.8)	0.341
Grade 1, n (%)	38 (67.9)	59 (65.6)	0.460
Grade 2/3, n (%)	5 (8.9)	7 (7.8)	0.517
Grade 3 post intervention, n (%)	53 (94.6)	86 (95.6)	0.546
<i>Trombus Grade- Sianos et al.</i>			
G2 small, n (%)	5 (8.9)	25 (27.8)	0.008
G3 medium, n (%)	11 (19.6)	56 (62.2)	0.001
G4 large, n (%)	30 (53.6)	8 (8.9)	0.001
G5 v. occlusion, n (%)	10 (17.9)	1 (1.1)	0.001
<i>Quantitative angiographic data</i>			
Lesion length, (mm)	20.7±2.1	20.1±3.2	/
Reference diameter, (mm)	2.7±0.5	2.7±0.2	0.579
MLD, (mm)	1.0±0.1	1.0±0.2	0.755
MLD post (in-stent), (mm)	2.6±0.3	2.7±0.2	0.290

Stent types			
BMS, n (%)	10 (17.9)	15 (16.7)	0.511
DES, n (%)	46 (82.1)	75 (83.3)	0.499
Multivessel intervention, n (%)	18 (32.1)	28 (31.1)	0.578

Data are means ± SD or n (%). BMI, body mass index, SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEF, left ventricular ejection fraction; MLD, minimum luminal diameter; BMS, bare metal stent; DES, drug-eluting stent.

Figures

Figure 1

Taxonomic Changes in Hyperglycemic Patients with STEMI. Phylogenetic tree showing differentiating taxa at the genus level between hyperglycemic and normoglycemic STEMI patients. The majority of depleted taxa in hyperglycemic microbiomes (red). Green indicates taxa enriched in hyperglycemic microbiomes.

THROMBUS MICROBIOTA

Figure 2

Thrombus Microbiota analysis. Panel A) filum, Panel B) genus, C) species in stool samples from hyperglycemic and normoglycemic STEMI patients. Panel D) regression analysis between admission blood glucose levels and thrombus Prevotella contents.

IN VITRO STUDY

Figure 3

In vitro study. Panel A), *Prevotella* growth curve in normal and high glucose concentration culture. Panel B), *Alistipes* growth curve in normal and high glucose concentration culture. * $P < 0.05$ and ** $P < 0.01$ vs 7 mM glucose concentration.

Figure 4

Regression analysis between thrombus Prevotella contents and thrombus dimensions (Panel A), TMAO thrombus levels (Panel B), CD40L thrombus levels (Panel C) and VWF thrombus levels (Panel D).

Figure 5

Risk adjusted Cox-regression analysis curves showing survival from MACE through 360 days in STEMI patients stratified by hyperglycemic vs normoglycemic patients (Panel A). Risk adjusted Cox-regression analysis curves showing survival from MACE through 360 days in STEMI patients stratified by thrombus dimension tertiles (Panel B). Risk adjusted Cox-regression analysis curves showing survival from MACE through 360 days in STEMI patients stratified by thrombus Prevotella content tertiles (Panel C). Risk adjusted Cox-regression analysis curves showing survival from MACE through 360 days in STEMI patients stratified by thrombus TMAO content tertiles (Panel D).

Figure 6

Hypothesis. 1) hyperglycemia promotes the growth and systemic colonization of Prevotella; 2) Prevotella overabundance increases TMAO levels; 3) high TMAO levels increase systemic insulin resistance; 4) increased insulin resistance increases hyperglycemia. Both hyperglycemia and high TMAO levels may increase CDL40 and VWF thrombus levels

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