Assessing Composition of Coronary Thrombus in STEMI Patients
A Multiscale Approach to Charaterize Samples Obtained by Catheter Aspiration

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Abstract: Percutaneous catheter thrombectomy allows collecting coronary thrombi from patients with ST-segment elevation myocardial infarction, preserving most of the sample characteristics available for both visual assessment and compositional analysis. This study aimed at identifying the association between thrombus macroscopic aspect and composition and, secondly, correlations between composition and ischemic time. Aspirated thrombi were grouped into "white", "red" or "mixed" according to their macroscopic appearance. Platelets, RBCs and fibrin were quantified on thrombus surface by SEM and on thrombus sections by a modified Carstairs histochemical staining. Fifty-three samples from 51 patients were included. The median [inter-quartile range] ischemic time was 210 [190-265] min. Seven (13%) "white", 19(36%) "mixed" and 27(51%) "red" thrombi were macroscopically identified. Median thrombus composition assessed by SEM was 23[11-41]%, 43[26-62]%, and 24[11-37]% for platelets, RBCs and fibrin respectively. Median histological analysis was 10[5-26]%, 45[31-64]% and 30[18-49]% for the same components. Significant difference in composition were found between "white" and "red" thrombi, showing respectively a higher amount of platelets ($p=0.003$) and fibrin ($p=0.007$). No significant correlations were found between composition and ischemic time suggesting that plaque instability and thrombus formation can occur before the symptom onset.

1 INTRODUCTION

Thrombus aspiration (TA) is a recommended technique in the treatment of myocardial infarction (MI) allowing thrombus removal from the culprit artery via a specific catheter (Keeley, 2003; Van der Werf, 2008, Steg, 2012). A reduced mortality in patients affected by myocardial infarction with ST-segment elevation (STEMI) has been associated to the use of TA in conjunction with primary percutaneous intervention (PCI) (De Luca, 2008; Vlaar, 2008; Burzotta, 2009).

TA allows collecting the biological aspirate in a minimally invasive way, preserving most of the original thrombus characteristics that are available for direct visual assessment and complementary laboratory analysis. The retrieved thrombus can varies in size, shapes and colour, giving readily available information to the interventional cardiologist (Quadros, 2012). Histological methods are also available for the characterization of thrombus morphology and for assessing thrombus age (Kramer, 2008) and scanning electron microscopy (SEM) has been proposed as a valid method for assessing composition of aspirated material (Silvain, 2011). These methods have the potentials of tracing the evolution of the pathology from the plaque rupture to the vessel recanalization. However, histopathological and microstructural analyses are time consuming and the macroscopic

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aspect of the thrombus remains the most accessible information to use at the bedside (Quadros, 2012).

We aimed at developing and applying a multiscale approach by integrating the macroscopic evaluation of the aspirated material with compositional data obtained from both histological and SEM analysis on a series of coronary thrombi collected during PCI with TA on STEMI patients. Data will be analyzed in order to identify firstly the association between macroscopic aspect of the thrombus and its composition and, secondly, possible associations between thrombus aspect and composition with ischemic time.

2 METHODS

Coronary thrombi were prospectively collected between November 2012 and November 2013 from all the STEMI patients admitted at the Division of Cardiology of the Santa Chiara Hospital in Trento, Italy, within 12 hours from the beginning of the MI symptoms and treated with catheter thrombus aspiration (Export 6F, Medtronic, Santa Rosa, California) before PCI. Procedures were performed according to the ESC guidelines (Steg, 2012).

Aspirated thrombi were collected on the disposable filter, washed with sterile saline to remove excess of blood and immediately fixed in 2.5% glutaraldehyde in 0.1M phosphate-buffered solution. Specimens were then stored in the fixative solution at 4°C until they were analyzed. Demographics, risk factors, blood biomarkers on admission, ischemic time (defined as the time lag between the onset of the AMI symptoms and the reperfusion therapy), and antithrombotic treatment were recorded on a data collection form.

2.1 Macroscopic Evaluation

In order to make the macroscopic evaluation of the aspirated material standardized and blinded to patients' characteristics and clinical conditions, thrombi were not evaluated immediately after aspiration. After a minimum fixation time of 48h, a photographic documentation of the aspirated material was performed by collecting one image per sample at 10x magnification with a stereo-microscope equipped with a colour digital camera under constant illumination conditions. Collected images were pooled and independently evaluated by two senior interventional cardiologists blinded to patient details and clinical characteristics. Aspirated thrombi were grouped into "white", "red" or "mixed" according to their macroscopic appearance and colour (Figure 1).

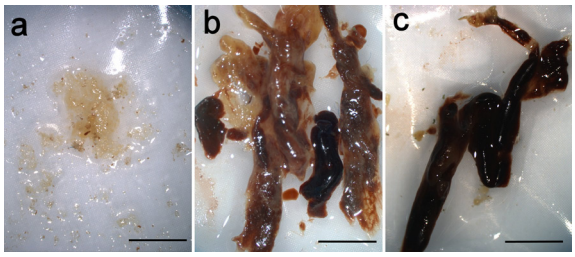

Figure 1: Macroscopic classification of aspirated coronary thrombi. Representative image of a "white" (a), "mixed" (b), and "red" (c) sample. Bar is 5 mm.

2.2 Scanning Electron Microscopy and Compositional Analysis

Before SEM analysis, samples were washed once in 0.1 M phosphate buffer and trice in distilled water, de-hydrated in ascending hydro-alcoholic solutions, dried in a laminar flow cabinet, mounted on a sample holder with double-sided conductive carbon tape, and sputter-coated with gold. SEM analysis was conducted on the thrombus surface with a XL30, ESEM FEG scanning electron microscope (FEI, Philips, Nederland).

To perform an operator-independent choice of the SEM fields of view, 20 point of interest were previously determined by superimposing a squared grid to the stereomicroscopic colour picture. A set of 20 electron-micrographs per each sample was than obtained in the same point of interest at a magnification of 2000x. The composition of the thrombus was determined by performing a semi-quantitative analysis of the SEM images, modifying the procedure previously proposed by Lucas et al. (Lucas, 2013). Briefly, a squared grid of 10μm element size was superimposed to each SEM image to obtain a total of 400 point of interest per sample. The observation of each point of interest allowed to quantify the following micro-morphological features: a) "platelets" (2 μm wide globules occurring as aggregates or adherent to the fibrin strands), b) "erythrocytes" (RBCs) (biconcave disc-shaped cells of about 7 μm in diameter), c) "fibrin" (fibrin network or fibrin fibres), d) "other" (other morphologies not included in the previous features). Representative SEM micrographs of the different micro-morphological features are reported in Figure 2.

SEM features quantification was considered feasible if "other" occurred in less than 50% of the investigated points. After the exclusion of points

associated to "other", the percentage of occurrence for each feature of interest was finally computed for each thrombus.

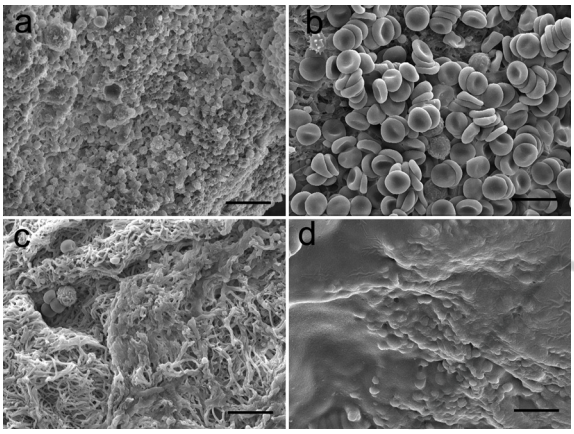

Figure 2: SEM images of the aspirated thrombus. Representative fields of view with a prevalence of platelets (a), RBCs (b), fibrin (c), and other features (d). Bar is 10 microns.

2.3 Histochemical Staining and Feature Quantification

After SEM characterization, thrombi were released from the carbon tape by adding a drop of absolute ethanol on the stub surface, carefully transferred on histological cassettes and re-hydrated by immersion in 70% ethanol in water for 24h and in distilled water for 48h. After this post-SEM conditioning, samples were processed for permanent histology by immersion in ascending hydro-alcoholic solutions, 100% ethanol and 100% xylene. After paraffin impregnation and embedding, 3 µm thick sections were cut with a rotary microtome.

Two consecutive sections per sample were stained respectively with haematoxylin-eosin stain (H&E) and a modified Carstairs stain (Figure 3). We adapted the original Carstairs staining method (Carstairs, 1965) to identify RBCs, platelets and fibrin in the thrombus sections. Staining times and fixation solutions were optimized for a better differentiation of thrombus components as described elsewhere (Lucas, 2014). Briefly, paraffin sections were hydrated, placed in 5% ferric alum for 10 minutes, rinsed in running tap water, stained in Mayer's haematoxylin for 5 minutes, and then rinsed again in running tap water. Sections were placed for 4 minutes in picric acid-orange G solution (20 mL saturated aqueous picric acid, 80 mL saturated picric acid in ethanol, and 0.2 g orange G) and then rinsed in distilled water. Sections were then placed in ponceau-fuchsine solution (0.5 g acid fuchsine, 0.5 g ponceau 2R, 1 mL acetic acid, and distilled water to 100 mL) for 2 minutes and then rinsed in distilled water. Sections were treated with 1% phosphomolybdic acid for 3 minutes, rinsed in distilled water, stained with aniline blue solution (1 g aniline blue in 100 mL 1% acetic acid) for 45 minutes, decoloured in 1% aqueous acetic acid for 3 minutes, rinsed in several changes of distilled water, dehydrated, cleared, and mounted with acrylic medium. On sections obtained from thrombi fixed for 48 hours or longer, Carstairs method produces differential staining of fibrin (bright red), platelets (grey-blue to navy), and RBCs (yellow) (Carstairs, 1965).

Platelet, RBCs and fibrin were quantified on Carstairs stained sections by setting specific colour thresholds in the L, a*, b* colour space (Figure 4). Pixel number for each feature was computed and percent occurrence over the whole section area was considered for the statistical analysis.

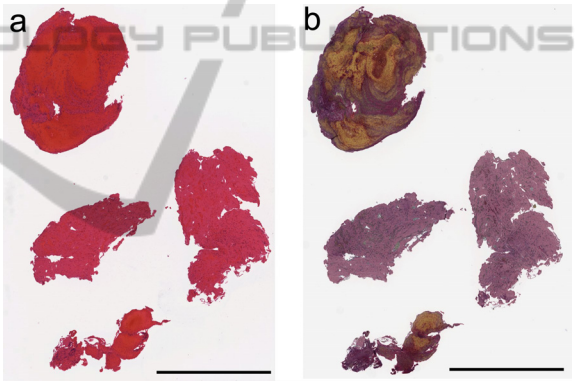

Figure 3: Histological features of a mixed thrombus. Consecutive sections stained with haematoxylin-eosin (a) and modified Carstairs stain (b). Bar is 1 mm.

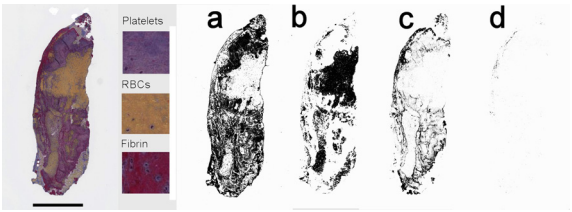

Figure 4: Histological analysis on a mixed thrombus section stained with Carstairs method (left). Representative colours associated to the three components of interest are shown. Binary images after specific threshold for platelets (a), RBCs (b) and fibrin (c). Inset d) reports about components not recognized in the three previous features and excluded from the calculation of compositional percentages. Bar is 1 mm.

Table 1: Patients characteristics.

Demographics	
Age, years	63.0 ±13.1
Male	41 (84.3%)
Risk factors	
Hypertension	58%
Active smoker	61%
Dyslipidemia	26%
Diabetes mellitus	13%
Familiarity	41%
Time delays	
Symptom onset to ASA, min	95 [50-140]
Symptom onset to ADP inhibitor, min	120 [90-180]
Symptom onset to PCI, min	210 [190-265]
Biomarkers on admission	
Troponin I, µg/ml	0.325±1.293
Platelet, mm^{-3}	252±82
CRP, mg/l	7.9±14.8
PT, INR	1.02±0.10
Creatinine clearance, ml/min	88.9±39.3
Antithrombotic treatment	
ASA	52 (98.1%)
ADP receptor inhibitor	53 (100%)
Clopidogrel	21 (39.6%)
Prasugrel	32 (60.4%)
GP IIb/IIIa inhibitor	25 (47.2%)
Pre-admission	0 (0%)
Catheterization laboratory	25 (47.2%)
Infarct related artery	
LAD	32 (60.4%)
RCA	17 (32.1%)
Cx	4 (7.5%)
CABG	0 (0.0%)

Values are mean ± SD, n (%), or median [interquartile range].
ASA : acetylsalicylic acid; ADP: Adenosine diphosphate; CABG: coronary artery bypass grafting; CRP: C-reactive protein; Cx: circumflex coronary artery; GP: glycoprotein; LAD: left anterior descending coronary artery; RCA: right coronary artery.

2.4 Statistical Analysis

Categorical variables were expressed as percentages. The results for normally distributed continuous variables are expressed as mean±standard deviation, and continuous variables that did not have a normal distribution are presented as median and inter-quartile interval (IQR).

The intra and inter observer correlations in the macroscopic analysis were assessed by weighted kappa coefficients considering that macroscopic categories were ordered from “white” to “mixed” and “red”. Kappa coefficient was interpreted as reported by Altman et al. (Altman, 1991).

The non-parametric Kruskal–Wallis test was used to compare three groups. If a significant difference was found with the Kruskal–Wallis test, then a Mann–Whitney U test was used to compare each pair of groups. Wilcoxon non-parametric test was used to compare distributions with paired data obtained from SEM and histological analysis. Categorical variables were compared using Fisher exact test. Spearman correlation coefficients were calculated to assess the relationships between platelets, RBCs and fibrin contents vs. ischemic time.

Multivariate linear regression analysis was performed to identify independent factors associated with the thrombus composition (age, sex and infarct related artery, antithrombotic treatment). All analyses used two-sided tests with a significance level of $p<0.05$. Data were analysed using STATA version 13.0 for Windows (STATACorp. College Station, TX, USA).

3 RESULTS

Sixty-three thrombi were obtained from 61 patients. Among them, six cases were excluded for incomplete clinical records and one sample failed histological preparation. Fifty-six aspirated thrombi were subjected to compositional analysis.

SEM characterization was not feasible according to the adopted criterion (“other” > 50%”) in 3 samples, leaving 53 samples from 51 patients who were eventually considered for reporting and statistical analysis. Patients’ characteristics are summarized in Table 1.

3.1 Thrombus Composition

Macroscopic classification gave 7(13%) “white”, 19(36%) “mixed” and 27(51%) “red” thrombi.

Intra-observer agreement was “good” (K= 0.785) with 84.9% of agreements. Inter-observer agreement was “moderate” (K=0.437) with 60.4% of agreements. “Red” and “white” thrombi were generally categorized in the same way by the raters, but discrepancies were frequent in evaluating mixed thrombi.

Features quantification on SEM images showed a median [IQR] composition of 23[11-41]%, 43[26-62]%, and 24[11-37]% for platelets, RBCs and fibrin respectively. At the histological analysis, the same samples showed a median [IQR] composition of 10[5-26]%, 45[31-64]% and 30[18-49]% for platelet, RBCs and fibrin respectively. Data obtained from SEM and histological methods were consistent and a paired non-parametric analysis showed no significant differences between the compositional distributions determined by the two methods.

To elicit possible associations between compositional data and macroscopic thrombus characteristics, results from SEM and histology were sub-grouped according to the macroscopic classification. Results are summarized in Figure 5.

Sub-group analysis of SEM compositional data showed a higher amount of platelets (p=0.003) and a lower amount of fibrin (p=0.007) in "white" thrombi in respect to "red" thrombi. No significant difference was found for the amount of RBCs within different macroscopic sub-groups, although a trend to higher median values of RBCs was found from "white" to "red" thrombi. Intermediate features were found for "mixed" thrombi but no significant compositional difference was present between "mixed" and "red" or "white" and "mixed" thrombi.

Differently from SEM compositional data, histological composition showed no significant compositional difference among the three macroscopic sub-groups.

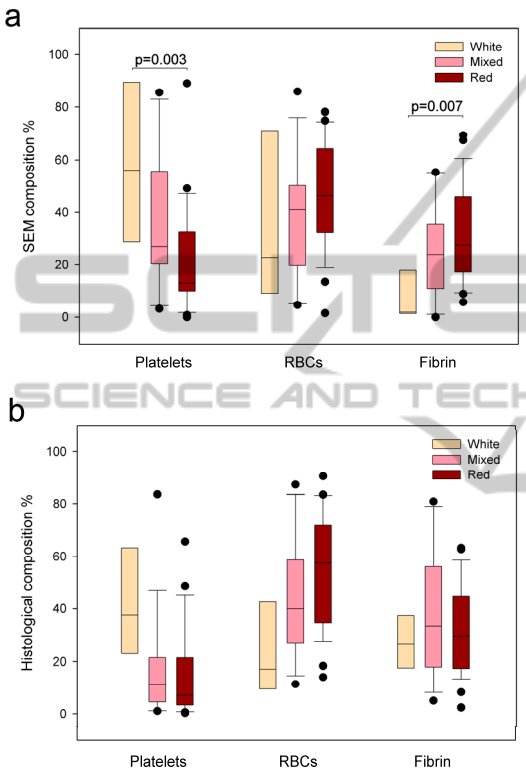

Figure 5: Composition of aspirated thrombi sub-grouped according to macroscopic categories. Values are indicated as percent of platelets, RBCs and fibrin determined by SEM (a) or histological (b) analysis.

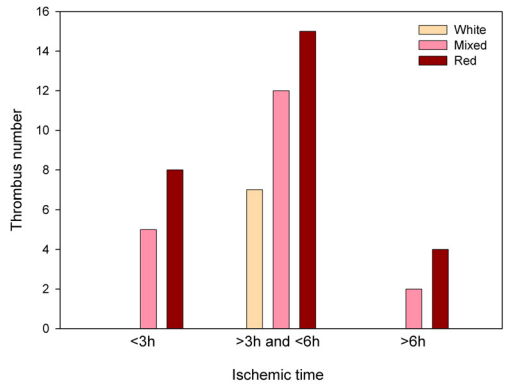

Figure 6: Number of aspirated thrombi (sub-grouped by macroscopic characteristic) plotted according to ischemic time.

3.2 Thrombus Composition Vs. Ischemic Time

Ischemic time for the 53 samples collected in this study ranged from 110 to 720 min with a median value of 210 min. According to previously proposed criteria (Silvain, 2011), ischemic time was categorized into three intervals (≤3 h, 3 to 6 h, ≥6 h). The number of "white", "mixed" and "red" thrombi grouped according to the ischemic time intervals is reported in Figure 6. No statistically significant difference was observed, but a large variability in ischemic time was associated to "red" thrombi.

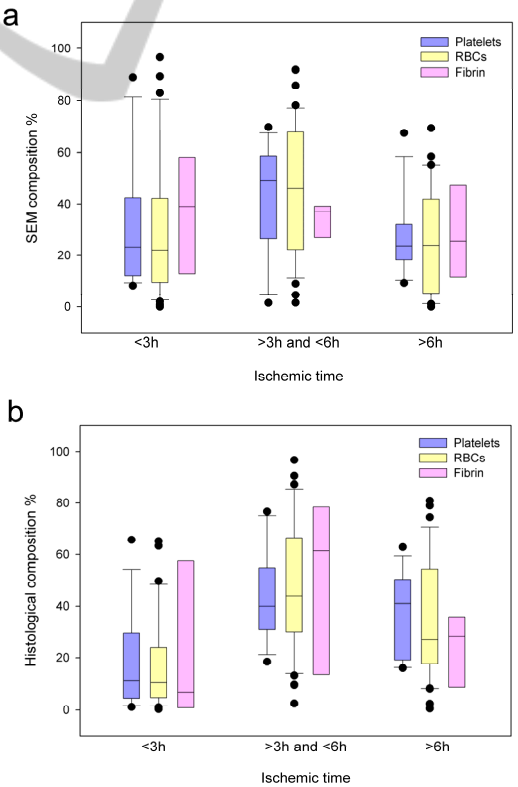

Figure 7: Composition of aspirated thrombi (percent of platelets, RBCs and fibrin) according to the three ischemic time intervals. Compositional data were obtained from SEM (a) and histological (b) methods.

Differently, all "white" thrombi were collected from patients with an ischemic time from 3 to 6 h.

The amount of platelets, RBCs and fibrin according to the three ischemic time intervals is reported in Figure 7 for compositional data obtained from SEM and histological methods. No significant difference in composition among the three sub-groups was found both on data obtained from SEM and from histology.

A univariate linear regression was performed on platelets, RBCs, and fibrin amount according to ischemic time, showing no linear association between composition and ischemic time.

A multivariate linear regression model was also performed to include in the analysis the potential confounding variables (age, sex, infarct related artery, risk factors, blood biomarkers, antithrombotic therapy) showing no significant effects on the thrombus composition.

4 DISCUSSION

The investigation of coronary thrombus characteristics and composition deserves high interest for defining the pathogenetic mechanism of acute coronary syndromes, for optimizing the therapeutic treatment according to thrombus characteristics and for having additional prognostic data on patients' mortality. Thrombi obtained by an aspiration device during the PCI treatment can give a rapid and informative feedback to the operator by the direct visual observation of the collected material. Moreover, the same biological aspirate is a good candidate for additional analysis on microstructure and composition. This work presents a new histological method using trichromic histochemical staining and colour image analysis for quantifying platelets, RBCs and fibrin on thrombus sections. The amount of the same features on the thrombus surface was also determined, in an independent way, by SEM and semi-automatic image analysis. Macroscopic features of the aspirated samples were also determined blindly to patients' clinical details by acquiring a low magnification picture under controlled illumination conditions. This approach represents a first example of integrated multiscale analysis that was not reported before in literature according to authors' knowledge. Results were obtained from 53 thrombus samples in a fully paired fashion, starting from a macroscopic qualitative classification to a quantitative determination of fibrin, platelets and RBCs by both histochemical and SEM methods.

Different macroscopic classifications were previously proposed to classify the colour of aspirated thrombi. Quadros et al. proposed a dichotomous classification into "white" and "red" thrombi (Quadros, 2012). They found that "white" thrombi occurred in one third of STEMI cases and were associated with lower ischemic time when compared with "red" thrombi (Quadros, 2012). Uchida et al. proposed a larger number of classes for the angioscopic in-situ classification, including also "transparent"," frosty glass–like", "light red", "mixed", and "brown" thrombi (Uchida, 2011). In accordance with other authors (Kirchof, 2003) and considering the inhomogeneities of colour within many aspirated samples, we considered three macroscopic categories, grouping thrombi into "white", "mixed" and "red". However, this classification showed a lower inter-observer agreement than those reported by Uchida and co-workers (Uchida, 2011). Differently from other studies where the macroscopic classification was performed during the PCI procedure, in this study the assignment of the macroscopic category was realized in a completely blinded fashion by evaluating only the low magnification image of the aspirated material captured with a digital camera. The difference in the agreement rate could be in part related with this methodology.

The compositional analysis was performed by a non-destructive SEM analysis of the outer surface of the thrombus. We adapted a previously presented protocol for SEM preparation (Silvain, 2011) in order to limit sample damage and allowing the subsequent histological analysis on the same aspirated material. Median compositional results obtained here were partly in agreement with those previously observed (Silvain, 2011). Specifically, we obtained a lower amount and fibrin and a higher amount of RBCs than those previously reported. Values for the amount of platelets are super imposable with those reported by Silvain et al.

Surprisingly, although RBCs in clots are the origin of the word "red" (usually referred as "old") thrombi as opposed to platelet-rich "white" (usually referred as "young") thrombi, we did not find significant differences in RBCs among different macroscopic sub-groups. Similarly, Silvain and co-workers failed to note a significant correlation between RBCs content and ischemic time by the SEM compositional analysis (Silvain, 2011).

Although the present study included a higher number of samples we did not evidence the same significant association previously reported between fibrin and platelets amount vs. ischemic time

(Silvain, 2011). In this study we included the total volume of retrieved fragments in both SEM and histological analysis. Differently, the compositional results of SEM analysis from Silvain et al. were limited to the main aspirated piece of the thrombus. This could partly explain differences in composition and time course between the two datasets, but the SEM exclusion criterion we applied could also have introduced a bias in sample selection.

Difficulties in evidencing significant differences in thrombus composition among the sample groups with different ischemic times could also be related to the inadequacy of the ischemic time in properly defining the thrombus age. Previous histopathological studies conducted on larger cohort of STEMI patients evidenced that intracoronary aspirated material by thrombectomy is frequently heterogeneous in terms of thrombus age (Rittersma, 2005, Kramer 2009). In 51% of cases, older thrombi (>1 day) were reported, which suggests an important discrepancy between the time of onset of the thrombotic process and the occurrence of acute clinical symptoms (<12 h in patients treated with TA) (Rittersma, 2005). The authors concluded that plaque instability frequently occurs days or even weeks before occlusive coronary thrombosis (Kramer, 2009).

The time between plaque rupture and thrombus formation is still unpredictable. Sudden coronary occlusion is often preceded by a variable period of plaque instability and thrombus formation, initiated days or weeks before onset of symptoms. The aspirated thrombus may be older than expected from the duration of the ischemic time (Kramer, 2009), and younger thrombus could be superimposed onto an older thrombus, thereby potentially confusing the observations.

5 CONCLUSIONS

A multiscale analytical approach to characterize samples obtained by catheter aspiration in STEMI patients has been realized by integrating qualitative information coming from the visual assessment of the coronary thrombus with compositional quantitative data obtained from histological and SEM analysis. Method here presented deserves high potential for understanding the mechanisms of thrombus formation in STEMI and for investigating correlations between composition and thrombus age.

Significant differences in composition were found, showing a higher amount of platelets and fibrin respectively for “white” and “red” thrombi. No significant correlations were found between composition and ischemic time, supporting previously reported data showing that plaque instability and thrombus formation can occur within longer time interval before AMI symptom onset.

Possible associations between thrombus composition determined with a multiscale approach and thrombus age assessed by histopathological methods should be investigated in future studies.

Eventually, specific analysis could be performed to elucidate potential association between thrombus composition and antithrombotic drug treatment.

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