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Acceleration of New Biomarkers Development and Discovery in Synergistic Diagnostics of Coronary Artery Disease

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1. Introduction

The current definition of biomarkers includes “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001)”. Accordingly, biomarkers are usually used for detection and establishing the magnitude of a physiological derangement as well as to monitor a treatment.

The role for imaging techniques and biomarkers in the diagnosis and treatment of myocardial infarction (MI) after percutaneous coronary intervention is well-established. Many candidate biomarkers emerging from genomics and proteomics research have the potential to serve as predictive indexes for guiding the development of interventional cardiology (Gerhardt et al. 1991; Katus et al., 1991; Lindpaintner, 1997; Kong et al., 1997). Among them the undisputed role still play cardiac proteins like troponins or creatine kinase-myocardial band (CK-MB) (Alcock et al., 2010; Lim et al., 2011). Less established, however, is the employment of biomarkers to determine long-term, progressive, or dynamic risk over time in patients with advanced coronary artery disease (CAD). Biomarkers offer a means to **track** differential exposure as well as **impact** of exposure. As such, they reflect individual vulnerability, ongoing person-environment interaction, and unmeasured environmental factors that mediate the effect of exposures (Fig. 1). Essential to a vision of **synergistic diagnostics** is a focus on the mechanisms of diseases. Understanding what is happening on a molecular and cellular level, how disease actually begins, how cells begin to express certain proteins, influence other cells and trigger processes (atherosclerosis, thrombosis, calcification) will allow to develop *in vitro* diagnostics and imaging techniques to distinguish these processes. By characterizing a comprehensive set of measurable processes that capture diverse pathogenic aspects of CAD, a real-time systems view of disease activity can be generated to improve decision making.

This chapter summarize a current view on the development of new biomarkers as a prognostic platform among patients at risk of CAD and upcoming complications.

Fig. 1. Model showing relationship of a biomarker with internal and external factors which have an impact on measurable and unmeasurable features of biomarker.

2. Bone remodelling biomarkers

About 10 years ago, the hypothesis that bone remodelling biomarkers might be involved in the progression of **coronary artery calcification** seemed to be tricky and beyond any reasonable expectation. However, in 1995 Boström *et al.* first time proposed the possible mechanisms for bone formation in artery walls involving retention of pluripotent cells or osteoblastic immigration coupled with embryonic-like osteogenic program (Boström *et al.* 1995). The main reason for understanding the regulatory mechanisms of vascular calcification was firstly related to therapeutic approaches to prevent and possibly reverse vascular mineralization (Demer, 1997; Parhami *et al.* 1997). The data from clinical studies regularly report an association between bone remodeling biomarkers and the presence, severity and progression of a broad range of cardiovascular diseases. Whether they are biomarkers or rather play a causal role in mediating or protecting against vascular injury is not clear. The mechanisms underlying the postulated role of bone remodelling biomarkers in atherosclerosis probably involve **inflammation** and **calcification** processes.

This section will focus on the prognostic significance of plasma bone remodelling biomarkers levels in stable and unstable CAD.

2.1 Biology of bone remodelling of biomarkers

Vascular biomineralization in an atherosclerotic plaque results from an imbalance in osteoblast- and osteoclast-like cells and the induction of vascular or immune cells differentiation into osteogenic cells (Demer & Tintut, 2008). Osteoblasts, osteoclasts and inflammatory cells are firmly involved in bone remodelling (Fig. 2).

Fig. 2. Bone remodelling osteoblasts and osteoclasts differentiation. Figure was produced using Servier Medical Art.

Fig. 3. The role of osteoprotegerin (OPG) in pre-osteoclast differentiation. OPG trap and neutralize a soluble receptor activator of nuclear factor kappa-B ligand (RANKL) which activates osteoclasts by its receptor (RANK). Figure was produced using Servier Medical Art.

Mesenchymal stem cells are precursors for **pre-osteoblasts**. **Osteoblasts** activity leads to bone formation and mineralization, their differentiation and activity is mostly regulated by RANKL (receptor activator of nuclear factor kappa-B ligand) inducers, such as: vitamin D (1,25[OH]₂D), glucocorticosteroids, parathormone (PTH), prostaglandins (PGE₂), lipopolysaccharides (LPS), histamine and pro-inflammatory cytokines: interleukins (IL-1 and IL-11), tumor necrosis factor alpha (TNF- α) and others (Eriksen, 2010). RANKL is a surface-bound molecule (also known as CD254). It is found on **osteoblasts** and serves to stimulate **osteoclasts** by RANK (receptor activator of nuclear factor kappa-B) activation and RANK/RANKL axis has a core regulatory role in osteoblasts and osteoclasts signalling (Fig. 3) (Caidahl et al., 2010).

2.2 Osteoprotegerin and osteopontin as risk factors of coronary artery disease

Several studies suggest the involvement of bone remodeling biomarkers in coronary artery disease and related atherosclerotic disorders (Van Campenhout & Golledge, 2009; Venuraju et al. 2010). Prime regulators of bone remodelling, such as osteoprotegerin (OPG) and osteopontin (OPN), are significantly and independently associated with inflammatory processes and arterial hypertension and may exert substantial influence on the severity of cardiovascular disease. (Stępień et al., 2011)

OPG is a soluble glycoprotein widely expressed in most human tissues including the bone (osteoblasts) and the vasculature (endothelial and vascular smooth muscle cells, VSMC) (Collin-Osdoby, 2004; Schoppet et al., 2002) that is implicated in the regulation of bone and vascular calcification. OPG is a member of the tumor necrosis factor (TNF)-related family and a part of the OPG/RANKL/RANK triad. OPG acts as a soluble secreted decay receptor for a receptor activator of nuclear factor kappa-B ligand (RANKL) and neutralize this essential cytokine required for the osteoclasts differentiation (Hsu et al., 1999) (Fig. 3).

RANKL expressed on osteoblastic, stromal and T cells binds to RANK (osteoclast differentiation factor) on the surface of osteoclasts, monocytic and dendritic cells and mediates a cell-to-cell signal responsible for osteoclastogenesis (Yasuda et al. 1998). Additional roles in immunological responses include the RANK-RANKL binding between dendritic and T cells which enhances the immunostimulatory capacity of dendritic cells and T cell proliferation (Green & Flavel 1999).

It was observed that *opg*-knockout mice (OPG $-/-$) develop early onset osteoporosis and arterial calcification (Bucay et al., 1998) and the restoration of the gene prevented osteoporosis progression and arterial calcification (Min et al., 2000). Increased OPG level has been observed in men with advanced CAD and plasma OPG level has proved to be an independent predictor of myocardial ischemia in asymptomatic diabetic patients (Avignon, 2007; Schoppet et al., 2003). Moreover, increased OPG has been related to the number and vulnerability of plaques as well as in carotid artery (Kadoglou et al., 2008; Vik et al. 2010) or coronary vessels (Palazzuoli et al., 2008), which suggests its involvement in the coronary disease progression (Mikami et al., 2008; Pedersen et al., 2010). Elevated OPG in plasma is univariable predictors of coronary artery calcification (CAC) progression (Anand et al., 2007). The sensitivity of OPG for detecting of CAC score higher than 200 Agatston units was 80% in patients with predialysis diabetic nephropathy (Schoppet et al., 2003). However, in the large Norwegian study by Pedersen *et al.* (Pedersen et al., 2010), adjustment for conventional risk factors attenuated the risk estimates for OPG levels. Only the subgroup of patients with stable angina pectoris (SA) with levels above the 90th percentile was at risk all-cause mortality: 1.94 (1.18, 3.18), $p=0.01$; CAD mortality: 2.29 (1.16, 4.49), $p=0.02$; and MI: 1.76 (1.02, 3.06), $p=0.04$.

In patients with acute coronary syndromes (ACS) the baseline OPG concentrations were strongly associated with increased long-term mortality (hazard ratio [HR] for log transformed OPG level 1.7 [range 1.5 to 1.9] $p < 0.0001$) and heart failure hospitalizations (HR 2.0 [range 1.6 to 2.5]; $p < 0.0001$) but weaker with recurrent MI (HR 1.3 [range 1.0 to 1.5]; $p = 0.02$) and not with stroke (HR 1.2 [range 0.9 to 1.6]; $p = 0.35$). The association remained significant after adjustment for conventional risk markers (Omland et al., 2008). In apparently healthy individuals (the European Prospective Investigation into Cancer in Norfolk – EPIC Norfolk cohort) high serum concentrations of OPG and soluble RANKL were associated with an increased risk of future CAD (Semb et al., 2009). OPG showed a significant association with the risk of future coronary events in both sexes. This association remained statistically significant after adjustment for traditional cardiovascular risk factors (i.e. age, diabetes, systolic blood pressure, smoking, total cholesterol and HDL cholesterol).

OPN is secreted as a calcium-binding glycoprophosphoprotein that has been implicated in bone remodeling and inflammation as well. Similarly to OPG, osteopontin is widely distributed in different human cells including osteoblasts, lymphocytes, macrophages, endothelial cells and vascular smooth muscle cells (Brown et al., 1992).

OPN is a cytokine and has the ability to stimulate migration of macrophages and osteoclasts (Giachelli et al., 1998; Suzuki et al., 2002) and proliferation of osteoclasts and vascular smooth muscle cells (Giachelli et al., 1998; Liaw et al., 1994). A growing body of experimental evidence suggests that OPN overexpression plays an essential role in modulating compensatory cardiac fibrosis and hypertrophy (Xie et al., 2004; Singh et al., 2010). OPN acts through different integrins and thus has a great potential to regulate populations of different cells on the molecular and cellular levels (Bazzichi et al., 2009; Burke et al., 2009). OPN plays a pivotal role in inflammation and atherosclerotic plaque formation in an animal model (Scatena et al., 2007). Recent data has indicated a high predictive value of OPN for secondary manifestations of atherosclerotic disease (e.g. cardiovascular death, myocardial infarction, stroke, and endovascular interventions) in a 3-year follow-up of patients undergoing carotid surgery (de Kleijn et al., 2010).

Baseline levels of OPN are independent predictors of future adverse cardiac events in patients with chronic coronary syndrome (CCS), and may be useful for risk stratification (Minorette et al., 2006). Recent data have indicated a high predictive value of OPN for secondary manifestations of atherosclerotic disease (e.g. cardiovascular death, MI, stroke and endovascular interventions) in a 3-year follow-up of patients undergoing carotid surgery. In a prospective study by Gogo et al. (Gogo et al., 2006), the association between angiographically quantified coronary artery calcification and OPG was not found. Detection of coronary calcification by coronary angiography may underestimate the calcification burden, thus synergistic diagnostics of coronary calcification should utilize more sensitive techniques of MSCT (Willemsen et al., 2009). However, in patients with CAD undergoing percutaneous coronary intervention (PCI) the highest OPN levels were associated with both plaque progression and restenosis in a stent ($p = 0.003$). In addition, OPN, IL-6, and CRP were higher in patients with ACS than in those with CCS (analysis of variance: $p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively) (Mazzone et al., 2011).

A question arises as to whether peripheral vascular function (calcification markers) matches the coronary arteries (calcification) and thus, whether it may serve as a surrogate marker to identify individuals with increased hazard of CAD and mortality (de Kleijn et al., 2010; Lieb et al., 2010; Scatena et al., 2007). Therefore bone-matrix proteins combined with cardiovascular imaging could be potential markers for vulnerable coronary artery plaques.

3. Microparticles

Microparticles (MP) are sub-micron sized cell membrane/cytoplasmic fragments that are released from the cell surface. There are two well-known cellular processes that can lead to the formation of MPs: chemical and physical cell activation (by agonists or shear stress, respectively), and apoptosis (Jimenez et al., 2003). However, the mechanisms that take place during MP formation are still not revealed. It seems that, the flopping of phosphatidylserine (PS) to the outer layer of the plasma membrane is pivotal. Finally, this process leads to the formation and shedding of MPs from activated or apoptotic cells. In resting condition the membrane asymmetry is maintained by an aminophospholipid translocase with **flippase** activity. Bilayer asymmetry is disrupted in the consequence of the inhibition of flippase activity by calcium influx. Increased calcium ions concentrations activate calcium-dependent calpains, which disturb cytoskeleton, promote the shedding of MPs (Morel et al., 2011) and stimulate **scramblase** and **floppase** activities, which lead to the collapse of the membrane asymmetry (Freyssinet & Toti, 2010).

MPs are qualitatively and quantitatively diverse and vary in diameter between 0.1 and 1.5 μm and may harbor a number of cell surface proteins (Fig. 4). MPs are released from various cell types such as circulating blood cells (platelets, lymphocytes T and B, monocytes and erythrocytes) and cells of the vessel wall (endothelial and smooth muscle cells) (Amabile et al., 2010).

Fig. 4. A platelet microparticle is carrying not only specific membrane adhesion proteins (P-selectin, integrins – e. i. GPIIb/IIIa,), but also may harbour and transfer tissue factor (TF) which has its procoagulant potential and other functional effectors (E-selectin, von Willebrand factor, arachidonic acid, thromboxane A₂), that can regulate aggregation, adhesion molecule expression, cell proliferation, apoptosis and endothelial migration. MPs may capsule messenger molecules (miRNA, DNA ?), cytokines, growth factors and calpains. Figure was produced using Servier Medical Art.

MPs from numerous cellular sources have been described in human plasma. They have received increasing attention as potential biomarkers of cell damage and activation or biovectors in blood coagulation, inflammation and cancer (Benameur et al., 2009; Hoyer et al., 2010). In several pathological states like dilated cardiomyopathy, chronic renal failure or cerebrovascular disease, MPs were used as biomarkers to identify a disease or to detect complications linked to a given disease (Bulut et al. 2011; Faure et al., 2006; Jung et al., 2009). Numerous clinical studies have evaluated their usefulness in the stratification of patients at risk for vascular disorders and to monitor response to treatment. Circulating MPs may serve as a marker for cardiovascular events in CAD patients or as a predictor of acute allograft rejection after heart transplantation (Morel et al., 2008; Sinning et al., 2010).

3.1 Microparticles discrimination and enumeration

The high level of microparticles' diversity may create a problem with compatible measurement of MPs using different analytical methods. The number of microparticles depend on the detection technique and a wide range of pre-analytical variables, i.e. blood collecting and handling, plasma preparation and storage conditions. Therefore, optimization and standardization of detection methods are important to define microparticles correctly and to avoid falsely high or low quantification. Even minor protocol changes significantly affected MP levels (Ayers et al., 2011).

3.1.1 Flow cytometry in MP analysis

Several research have evaluated the impact of these different parameters to propose a pre-analytical protocol for MP analysis. Three ISTH Scientific and Standardization Subcommittees (SSC Vascular Biology, DIC, and Haemostasis & Malignancy) have initiated a project aimed at standardizing the enumeration of cellular MPs by means of flow cytometry method (FCM). The first collaborative workshop was set to establish the resolution and a threshold levels of the flow cytometers currently used in laboratories. Additionally, the interinstrument reproducibility of platelet MP enumeration in human plasma was analyzed (Lacroix et al., 2010). The study included 40 laboratories and 59 flow instruments were validated according to the protocol based on Megamix beads calibration to discriminate microparticles between 0.5 μm and 0.9 μm using the forward scatter (FS) channeling (FSC) parameter (FS/FSC). After that, selected laboratories received PFP samples prepared as frozen aliquots by the core laboratory, to avoid any preanalytic-linked variability. The authors found high discrepancy among Becton Dickinson instruments, as well within low, medium and high values of MP: coefficients of variation were 78%, 60% and 91%, respectively. Whereas interlaboratory reproducibilities were 30%, 15% and 17% for low, medium and high values among Beckmann Coulter instruments. These data indicate that standardization of platelet MPs enumeration by FCM depends on intrinsic characteristics of instruments. Moreover, standardization by calibrated beads such is useful tool for MP enumeration, however, calibrated beads do not reflect real condition of MPs in human plasma.

3.1.2 Indirect methods for MP enumeration

Alternative methods for MP enumeration based on TF-activity/antigen or platelet glycoprotein GPIIb-integrin have been already described (Huise et al, 2009; Kuriyama et al., 2010). The activity of tissue factor is evaluated using a chromogenic substrate for factor Xa,

thus the ability of MPs to promote factor X activation in the presence of factor VII using a chromogenic activity assay is utilized (Huisse et al, 2009). Alternatively, TF antigen or activity can be measured in plasma or whole blood (Key NS & Mackman N, 2010). However, determination of microparticle size is not possible by such approaches.

3.1.3 Pre-analytical variability in MP determination

The analysis of different protocols used in MP preparation showed that washing, centrifugation, filtration of buffer and long-term freezing influenced significantly the MP quantification (Ayers et al., 2011; Dey-Hazra et al., 2010). Freezing samples at -80°C decreased MP levels (Ayers et al., 2011; Shah et al., 2009). The second collaborative workshop was dedicated to propose a common pre-analytical protocol useful for standardization of pre-analytical variables in determination of MPs (Scientific and Standardization Committee 2010).

3.1.4 Specific antigens in MP discrimination

There are two main features of native MPs: the small size and the anionic phospholipid - PS on the outer leaflet of their membrane. In addition, MPs carry surface membrane antigens reflecting their cell of origin, including those induced by cellular activation, cell injury or apoptosis. These properties permit detection of specific subpopulations, such as endothelial, leucocyte or platelet-derived MPs (Diamant et al., 2004).

PS is specifically bound to annexin V and is recommended as a distinguish marker for MP enumeration (Bulut et al., 2009; Shah et al. 2009). However, a number of evidence suggests that some vesicles derived from endothelial cells are PS-negative by annexin-V labelling (Jimenez et al., 2003; Sekula et al., 2011). In platelet-poor plasma obtained from healthy donors, 80% of platelet-derived MPs failed to bind annexin V (Connor et al., 2010). In this case, a phalloidin-staining of actin filaments could be helpful in discrimination of MPs and other cell fragments (Mobarrez et al., 2010). Washing samples as well as double centrifugation result in decreased annexin-V (Ayers et al. 2011).

3.1.4.1 Platelet MPs

Platelets constitute the main source of circulating procoagulant MPs under many physiological and pathophysiological situations (Geiser, et al., 1998; Huisse et al, 2009; Kuriyama et al., 2010). Procoagulant platelet derived MPs are enriched in P-selectin (CD62P), cell surface protein (CD63), integrins: GPIIbIIIa ($\alpha 2b\beta 3$), GPIIb ($\alpha 2b$, CD41), GPIIIa ($\beta 3$, CD61) and GPIb (CD42b), tissue factor (CD142, TF) or calpains (Figure 4).

Patients with unstable angina (UA) and AMI had a significantly increased number of procoagulant MPs: GPIIbIIIa-positive, CD62P-positive and CD41-positive (Huisse et al., 2009; Morel et al., 2004; Stankiewicz et al., 2007; van der Zee et al. 2006). The total number of platelet-derived MPs were numerically higher in patients with no recanalisation compared to patients with recanalisation (Huisse et al., 2009). However, we observed paradoxically lower number of CD62P-positive platelets in whole blood obtained from patients with ACS, than from SA patients, but the level of soluble P-selectin in plasma was significantly higher than in those with ACS (Figure 5). We may suspect that soluble P-selectin levels are derivatives of platelet origin MPs (Chung et.al., 2009).

3.1.4.2 Tissue factor-bearing MPs

It was shown by cell sorting with the specific marker CD42b that under resting conditions, blood-borne TF was mainly harbored by platelet-derived MPs (Müller et al, 2003). In acute

coronary syndromes, TF triggers the formation of intracoronary thrombi following endothelial injury, activation of macrophages and apoptosis of smooth muscle cells (SMCs) and macrophages (Morel et al, 2006). Apoptotic (annexin V-positive) MPs support a number of TF-positive MPs from different origin. Apoptotic macrophages and SMCs are the main source of membrane-bound TF and they contribute to TF accumulation. Formation of TF triggering MPs rich in PS provides a suitable anionic phospholipid surface for assembly of the tenase and prothrombinase complexes and thrombin activation (Del Conde et al., 2005).

Fig. 5. Platelet activation measured as a percentage of surface P-selectin-positive (CD62+) platelet (PLT), and by monocyte/platelet aggregates (MO/CD61+) and neutrophil/platelet aggregates (N/CD61+) in peripheral blood from patients with stable angina (SA) and acute myocardial infarction (AMI), and by levels of soluble P-selectin in patients with stable angina (SA), and acute myocardial infarction (AMI). Data are expressed as medians. * $p<0.05$, *** $p<0.00001$ for the comparison.

Additionally, an increased number of TF-positive (CD142-positive) MPs in patients with ACS was observed (Figure 6) (Huisse et al., 2009; Steppich et al., 2005). Moreover, elevated levels of different origin TF-bearing MPs were significantly higher within the occluded coronary artery than in peripheral blood samples (Morel et al. 2009). It suggests their contribution in coronary atherothrombosis and *in situ* formation of procoagulat MPs.

3.1.4.3 Endothelial microparticles

Endothelial microparticles (EMPs) are an emerging marker of endothelial cell (EC) activation and dysfunction and their circulating numbers are elevated in a number of pathologic states including cardiovascular disease. Many studies suggest that endothelial

cell-derived MPs have a paracrine role and contribute to the development of endothelial dysfunction in most cardiovascular diseases: CAD, ACS, MI, hypertension and congestive heart failure. Moreover, diabetes, end-stage renal failure and pulmonary or venous embolism are strong factors bringing about EMP shedding [Bal et al., 2010; Chirinos et. al., 2005; Faure et al., 2006; Morel et al., 2004a]. In this case patients have marked activation of endothelial, platelet, and leukocyte MPs.

Endothelial-derived microparticles (EMPs) may carry different endothelial originating coagulation factors, for example TF, which contribute to the clot formation and lysis (Chou et al., 2004; Stępień et al., 2007b). Patients with AMI displayed higher levels of all MPs than patients with SA and CD31-positive EMPs appeared the main source of procoagulant MPs (Morel et al., 2004b). In patients with ACS significant correlations between both the total

Fig. 6. Representative dot plot of circulating microparticles (MPs) in a patient with acute coronary syndrome (ACS) and in a control voluntary. A, C - flow cytometry gating logic, MPs were initially gated by forward (FCS-H) and side scatter (SSC-H) in logarithmic scale; B, D - fluorescence plots show MPs binding of annexin V-FITC (FL1-H) and anti-CD142-PE (FL2-H) monoclonal antibody.

number as well as the level of CD34, CD51 and CD142 were observed (Stankiewicz et al., 2007). Moreover, increased number of EMPs (E-cadherin/CD144-positive MPs) was an independent predictor of future cardiovascular events (HR 2.42 [range 1.03 to 5.68], $p=0.04$), but not for all-cause mortality (HR 2.10 [range 0.83 to 5.32] $p=0.12$) in patients with heart failure (Nozaki et al., 2010) and the assessment of EMPs improved prediction of future cardiovascular events in patients with CAD (Nozaki et al., 2009).

4. Clotting

Clotting is a rapid and highly dynamic process, which involves both platelets and coagulation factors. To monitor the clotting process a lot of instrumentations and methods are engaged: i) clotting times: the activated partial thromboplastin time (aPTT) and the prothrombin time (PT); ii) thromboelastography; iii) assessment of thrombin generation markers and thrombin inhibitors; iv) the real-time monitoring of thrombin generation. This section will focus on the prognostic significance of thrombin generation markers in stable and unstable CAD.

4.1 Markers of thrombin generation in CAD patients

Antithrombin (AT) appears to be the most important stoichiometric inhibitor which forms equimolar complexes with thrombin molecules – TAT (thrombin-antithrombin) complexes. A concentration of TAT complexes measured in peripheral venous blood and in blood collected at the site of microvascular injury reflect thrombin generation. TAT complexes are expressed during clot formation and there are (alike fibrinopeptide A and F 1+2 fragments) markers of thrombin activation (Pelzer et al., 1988; Pelzer et al., 1991). These markers are elevated in pro-thrombotic conditions. In patients with cardiovascular disease, the detection of a prothrombotic state may have two major implications: i) to extend the duration and ii) to monitor the dose of anticoagulation after cardiac intervention. The thrombin plasma activity is very firmly associated with CAD.

The potential coagulation activity in plasma can be evaluated by the rate of thrombin formation and the total amount of formed thrombin is measured by means of chromogenic or fluorescence methods (Devreese et al., 2007; Hemker et al., 2002). This thrombin potential in plasma can be assessed by different methods and the Calibrated Automated Thrombogram (CAT) applies a fluorogenic substrate. A chromogenic substrate is used in Behring Coagulation System (BCS). In both methods thrombin generation is activated by diluted recombinant tissue factor (TF), but in the BCS method a non-defined fibrin aggregation inhibitor is present. Both methods are applied in diagnostics. In CAT a calibration factor is measured in a plasma sample identical to that in which thrombin generation is being determined and the course of the calibration factor is assessed during the entire measurement (Figure 7). Thrombin generation assays seem to be useful in endogenous TF assessment (Ollivier et al. 2010; Stępień et al., 2007a).

4.2 Blood sampling for coagulation markers assessment

The most important think in coagulation diagnostics is to apply a reliably sampling method. To ensure accurate measurement samples must be collected in the circumstances under which false elevations of molecular markers of hemostatic and fibrinolytic activation will not occur. Thus, atraumatic antecubital venipuncture into vacutainer containing buffered sodium citrate is essential and the contamination with calcium or magnesium should be

avoided (van den Besselaar et al., 2007; Stegmar et al., 2007). To avoid activation of coagulation by tissue thromboplastin, each collection of citrated plasma should be preceded by a serum tube. Duration of needle puncture, rather than duration of tourniquet use, produced the greatest elevation in plasma levels of TAT and F1+2 (Omote et al., 2008).

Fig. 7. The rate of thrombin formation is presented as the thrombin concentration against time curve. Three parameters are presented: lag time (T_{lag}), peak height (C_{max}) and endogenous thrombin potential (ETP).

4.3 Prognostic value of thrombin generation in cardiac events

Increased circulating levels of thrombin and its markers characterize ACS (Ardissino et al., 2003; Takano et al., 1991). Plasma F1+2, normally about 1 nM, is roughly 1.5-2-fold higher than observed in SA patients, reaching maximum values in AMI (Ardissino et al., 2001). U-shaped relationship between plasma prothrombin fragment 1+2 levels and the risk of developing cardiac death or renewed myocardial infarction was observed. Intermediate levels (1.5-1.9 nM) were associated with the lowest risk, whereas both higher (>1.9 nM) and lower (<1.5 nM) values were associated with an increased risk (RR 1.56 [range 1.25 to 2.28] and RR 1.35 [range 1.11 to 1.86], respectively) (Ardissino et al., 2003). Hypercoagulable state measured as thrombin-antithrombin complexes (TAT) levels and as calibrated automated thrombogram reflects vascular impairment in CAD patients (Stępień et al., 2007a). It was observed that high TAT levels may predict mortality in chronic heart disease group after adjustment for classic risk factors (Marcucci et al., 2006). In empirical reconstruction, simulated maximum thrombin levels ($p<0.01$) and rates ($p<0.01$) were 50% higher with ACS while the initiation phases of thrombin generation were shorter than in patients with stable CAD (Brummel-Ziedins et al., 2008). Elevated levels of thrombin derivatives are associated with clinical risk factors for stroke (Lane et al., 1983; Takano et al., 1991). Elevated thrombin concentration reflects hypercoagulable state in patients with hypertension (Hoeper et al.,

1998; Kłoczko et al., 1996), hyperglycaemia (Undas et al., 2008) and hypercholesterolemia (Wada et al., 1992; Sanguigni et al., 2005; Undas et al., 2005).

5. Conclusion

Endothelial and platelets activation leading to cardiovascular complications can be evaluated quantitatively by measurement of plasma levels of circulating MPs. Moreover, a multiple biomarkers strategy that includes bone remodeling biomarkers (OPG, OPN) and clotting properties can provide better risk stratification of cardiovascular events. Development and discovery of new biomarkers may improve clinical assessment of patients who might benefit more from treatment. Synergistic strategies in diagnostics seem to be more advantageous than routine method in prognosis and patients' management.

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