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Letters to the Editor

Gelatinase concentrations and zymographic profiles in human breast cancer: Matrix metalloproteinases circulating in plasma are better markers for the subclassification and early prediction of cancer: The coagulation/fibrinolysis pathways alter the release, activation and recovery of different gelatinases in serum

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Dear Sir,

Somiari *et al*. recently published the measurements of serum levels and activities of matrix metalloproteinases (MMP) in patients classified as low and high risk (according to Gail score), benign disease or breast cancer.[1] We read their article with concern, which described that total, active and activity of MMP were significantly increased in serum of cancer patients respect to benign diseases and control groups, concluding that serum MMP may classify patients with breast diseases. Surprisingly, the authors have just published an almost overlapping study using plasma samples following the same research protocol,[2] reporting that also plasma MMP concentration and activity may permit subclassification of patients with breast disorders.

Although the role of proteinases in cancer initiation and progression has been on a roller-coaster ride for the past years, there is no doubt that among proteinases, MMP family members are involved in human breast cancer (BC).[3,4]

Among Matrixins, Ca/Zn-dependent proteinases ubiquitously involved in metabolism regulation,[5] MMPs are characterized by the ability to extensively degrade proteins, in particular almost all extracellular matrix proteins.[6] To prevent the vast potential of MMPs, crucial in physiological cellular mechanisms (*e.g*., cell development, organogenesis, tissue remodelling and repair, apoptosis)[7] from becoming destructive during pathological processes (*e.g.,* inflammation, tissue degeneration, tumor growth, invasion and metastasis),[8] a fine control is needed. The detrimental role of MMPs in cancer (not sufficiently counterbalanced by the endogenous specific inhibitors TIMP) has not been successfully blocked by the development of synthetic/natural MMP inhibitors that failed to limit the disease progression during several clinical trials.[9] Although, the role of MMPs in early stages of cancer remains to be clearly established, the increased expression of MMPs in cancer-related processes is also enhanced by the cross-talk signalling between stromal and inflammatory cells within the tumor microenvironment, leaving the way open for the multifaceted functions of proteinases in cancer development.[10]

No question is of greater interest to the clinicians dealing with breast diseases than the differentiation of BC subtypes through the determination of biomolecular markers useful for BC diagnosis and prognosis. Although the identification of the best source between plasma and serum for surrogate biomarkers is a matter of strenuous efforts and debate,[11–17] the scientific interest in the measurement of MMP in plasma/serum continues to mount, enhancing their promising development as reliable biomarkers.

The conclusions of Somiari *et al*.[1] about the usefulness of MMP in serum samples strongly contrasts with the growing evidence that plasma MMP-9 is considered as a better marker for early diagnosis, progression and prognosis of BC.[2,18,19] The faithful determination of MMP-2 and -9 circulating in blood depends on the procedure of blood sampling/handling, a methodological issue discussed extensively in this Journal[20] and analytical journals.[11–17] Although these articles unequivocally demonstrated the "artificially" higher levels of serum MMPs with respect to plasma, manuscripts ignore these statements and continue to publish based on the measurements of serum MMPs. A serious preanalytical flaw in this otherwise well-conceived study,[1] undermines confidence in the authors' conclusion, highlighting several caveats that are implicit, looking more correlative rather than mechanistic in nature.

To correctly interpret data and to avoid pitfalls or wrong expectations in future studies in BC using circulating MMP-2 and -9 as surrogate biomarkers, we would like to draw the attention of interested clinicians to the crucial role of blood sampling/handling when studying the functional involvement of MMPs in BC. We provide an explanation of why serum does not reflect the correct MMP expression, which should be the mirror of tumor microenvironment and not be due to the release by other cell sources, such as circulating white blood cells.

The stroma surrounding the mammary terminal ductal/lobular unit is extensively infiltrated by migrating blood cells, mostly macrophages and eosinophils, capable of producing factors (*e.g.,* angiogenic factors such as VEGF and angiopoietin, proteinases such as MMP-9 and uPA, growth factors such as EGF and CSF-1, cytokines and chemokines) that can promote both normal development (from postnatal morphogenesis through lactation) and escape of tumor cells from the local microenvironment, enhancing their metastatic potential.[21–23] Although the origin/function of white blood cells in BC are not yet fully elucidated,[22] an intensive interplay exists between breast tumor cells and leukocytes.[23,24] Accordingly, also the count of peripheral leukocytes strongly influences BC prognosis.[25] Platelets, eosinophils, neutrophils and monocytes contain high amounts of MMP-9 forms that may be extracellularly released upon activation or during aggregation.[26–28] It is worth noting that BC infiltrating lymphocytes showed an increased

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expression of several MMPs during invasion and metastasis, as well as mononuclear and neutrophilic infiltrates were associated with breast epithelial proliferation.[22–24] These evidences suggest the role of leukocyte degranulation for the release of MMP in blood, especially in patients affected by breast diseases.[21,25] In this respect, the use of anticoagulants[13–15] limits the mobilization of gelatinase-rich granules of human neutrophils,[27] leading to a minimal release of MMP-9 forms in plasma.[15,16] The major differences between plasma and serum involve MMP-9 forms and to a much lesser extent MMP-2, which is considered the constitutive MMP circulating in blood.[13,17] Somiari *et al.*[1] (reporting as "still unresolved" the preanalytical issue of blood sampling) are in strong contrast with a wide number of publications that extensively demonstrated (not only in analytic journals[11–17] but also in this[18,20] and other cancer journals[2,19]) the presence of MMP-9 forms in higher amounts in serum, not only related to the disease but mainly linked to coagulation/fibrinolytic pathways. In fact, the use of anticoagulants limits the release and activation of MMP-9 forms from blood cells.[14–16] Moreover, the rapid separation of serum (minimizing the time between blood drawing and centrifugation) does not avoid the artificially higher MMP-9 release in serum respect to plasma.[29] All the studies (carried out through quantitative ELISA assays and zymographic approaches) collide to the same final conclusion that plasma represents the best source of MMPs as circulating biomarker for cancerous diseases.

To further confirm these evidences, we report in Figure 1*a*, a gelatin zymography that clearly reveals the differences in circulating MMP-9 forms found in plasma, serum and nipple aspirate fluid from the same BC patient, demonstrating that serum contains higher concentrations and different zymographic profile of MMP-9 forms respect to plasma and breast secretion. Moreover, we would focus the attention about the sensitivity and specificity of gelatin zymography, an inexpensive and unique method allowing both the qualitative and quantitative discrimination between all the pro- and active-MMP forms.[31]

In addition to the interindividual differences in white blood cell count, that may strongly influence MMP-9 levels and activity in serum, both the coagulation and fibrinolytic mechanisms may alter MMP-9 release in serum respect to plasma. As depicted in Figure 1*b*, coagulation and fibrinolysis factors lead to an overall increase of MMP-9 in serum. During coagulation, thrombin may facilitate platelet aggregation and activation with subsequent secretion of pro- and active MMP-9.[32] Moreover, thrombin generates fibrin that binds the fibronectin-like regions present in all MMP-9 forms (*i.e.* 92, 130 and 225 kDa); fibrin-proMMP-9 complex is then processed via a plasmin-dependent pathway releasing active MMP-9 forms.[33] During fibrinolysis, urokinase-type plasminogen activator generates plasmin, which stimulates the secretion of both pro and activated MMP-9 forms from several white blood cells; accordingly, no active MMP-9 forms were detected in the absence of plasminogen, cathepsins and stromelysins.[30,34]

In conclusion, we want to focus the attention to limit the misuse of serum for MMP-9 determination in clinical studies, in particular on BC. Somiari *et al.*[1] reported increased serum MMP-9 levels and activity in more than 66% of patients studied respect to the results previously obtained in plasma[2] from patients enrolled with the same research protocol: we provide biological mechanisms explaining their contrasting results, reinforcing the concept that plasma is the best and more reliable source for MMP clinical studies. To avoid pitfalls, artefacts and misinterpretations of total and active MMP serum concentrations (because of leukocyte and platelet degranulation and protease activation), it is conceivable that quantitative measurement of gelatinases, in conjunction with qualitative zymographic profile, should be performed in plasma. It could represent a promising tool for improving breast cancer classification, diagnosis and prognosis,[35] a further weapon in the fight against human cancer.

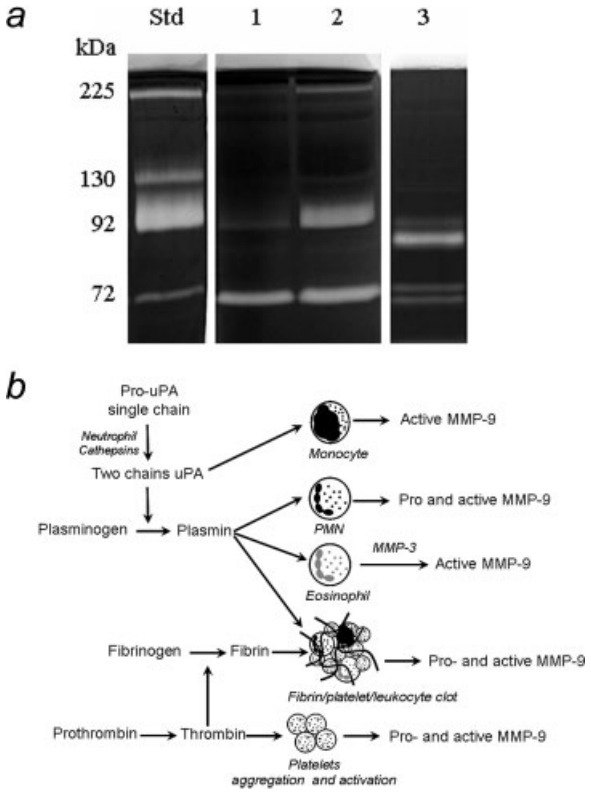

FIGURE 1 – Release of matrix metalloproteinases in serum and plasma samples. (*a*) Zymographic profile of gelatinase forms separated in plasma, serum and nipple aspirate fluid (lane 1, 2 and 3 respectively) from the same patients prior to biopsy proving the presence of breast cancer. *Std*, gelatinase standard of 72, 92, 130 and 225 kDa of molecular mass. The gelatin zymography was carried out according to previously detailed method.[13,15,30] (*b*) Scheme of MMP-9 form release and activation in serum, during both coagulation and fibrinolysis pathways. Blood leukocytes and platelets contain high amounts of proforms of matrix metalloproteinases that may be released upon activation into blood through the coagulation-induced degranulation, by active urokinase-dependent plasminogen activator and plasmin related mechanism. Intrinsic and extrinsic pathways of coagulation generate fibrin molecules which bind dose-dependently all the proforms of MMP-9, cleared as active form by plasmin activity. Moreover, thrombin activity may facilitate platelets aggregation and release of active MMP-9 forms. These biochemical mechanisms account for the increased concentrations of MMP-9 forms found in serum respect to plasma, in which the anticoagulants strongly limit MMP-9 recovery.

Yours sincerely,

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