immune disorders, inflammation, cancer, and cardiovascular disease (3). Such high concentrations of circulating adhesion molecules may be of interest for diagnostic and prognostic purposes, as demonstrated for ICAM-1 and P-selectin, which provide useful information in healthy individuals and in patients with cardiovascular diseases (9). However, reference values for healthy children and adults have not been defined.

In the current study, we measured serum ICAM-1 and E-_, P-_, and L-selectin concentrations and determined their age and sex-specific reference intervals over a wide age range (4–55 years) in a healthy population of both sexes.

Earlier studies dealing with serum adhesion molecule (ICAM-1 and E-selectin) concentrations in healthy pediatric populations pointed to an age dependency (10–12). In this study, we observed that mean serum ICAM-1 and E-_, P-_, and L-selectin concentrations steadily decrease during childhood. ICAM-1 and P-selectin concentrations did not vary with age in adulthood, whereas L-selectin decreased in both sexes and E-selectin increased in women only. Although the decrease in adhesion molecule concentrations with age in children has already been described, its physiologic significance during normal development is unknown. Authors of previous studies in adults observed that ICAM-1, E-selectin, and P-selectin concentrations did not vary with age between 18 and 65 years (3, 5, 8, 10, 13, 14), but the decrease in L-selectin during adulthood has not been described.

ICAM-1, E-selectin, and P-selectin concentrations display a significant sex dependency only in adults, with men having higher concentrations than women. These sex-related differences, as reported previously in healthy adults (8, 14–16), are probably partly attributable to steroid hormones, especially estrogen. Indeed, healthy postmenopausal women on hormone replacement therapy had lower concentrations of these adhesion molecules than controls not on replacement therapy (7, 17, 18), and in vitro studies have shown negative regulation of adhesion molecule expression by estrogen (19).

In conclusion, we have shown that serum ICAM-1 and E-_, P-_, and L-selectin concentrations are age-dependent in childhood and sex-dependent in adulthood. Application of age- and sex-adjusted reference intervals appears to be necessary. These findings emphasize the need to use age- (for children) and sex-matched controls (for adults) in all analyses of the possible clinical impact of circulating concentrations of adhesion molecules.

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References

Zymographic Analyses and Measurement of Matrix Metalloproteinase-2 and -9 in Nipple Aspirate Fluids, Ferdinando Mannello1 and Maurizio Sebastiani2 (1 Istituto di Istologia & Analisi di Laboratorio, Facoltà di Scienze Matematiche, Fisiche e Naturali, Università degli Studi “Carlo Bo”, Via E. Zeppi, 61029 Urbino-PU, Italy; 2 Centro di Sonoeglogia, ASL-1, 61100 Pesaro, Italy; * author for correspondence: fax 39-0722-322370, e-mail f.mannello@uniurb.it)

The matrix metalloproteinases (MMPs) are Ca2+/Zn2+ endopeptidases involved in extracellular matrix (ECM) degradation (1) in both tissue remodeling (2) and tumor growth and invasion (3). MMP-2 (gelatinase A; EC
NAFs (indicative of premalignant disease), "nonlactating women (age range, 34–68) medically or surgically treated during the previous year. Exclusions included patients reporting pregnancy or breast alterations within 3 years before the study or who were excluded because of pregnancy. Healthy controls.

In the series, 168 patients (n = 92) were diagnosed with infiltrating ductal carcinoma, 12 patients with infiltrating lobular carcinoma, and 23 women as healthy controls.

Of 115 women participating in a breast cancer (BC) prevention trial at the Centre of Senology (1998–2002), we excluded 30 patients reporting pregnancy or breast alterations within 3 years before the study or who were medically or surgically treated during the previous year. NAFs were prospectively collected from the remaining 85 nonlactating women (age range, 34–47 years): 45 patients with benign breast disease (BBD; diagnosed by echographic and/or mammographic approaches), 5 patients with BBD at the time of NAF collection who subsequently developed BC, 12 patients with infiltrating ductal carcinoma diagnosed by surgical biopsy, and 23 women as healthy controls.

NAFs (20–600 μL) were centrifuged at 19 000g for 20 min at 4°C, and after removal of the lipid layer, the supernatant was analyzed for protein and MMP content (26). Gelatin zymography was carried out on 7.5% polyacrylamide gels copolymerized with 2 g/L 90 Bloom Type A gelatin from porcine skin (Sigma) (32).

After electrophoresis, gels were washed in Triton X-100 (25 mL/L) and incubated for 24 h (37°C) in enzyme buffer (containing, per liter, 50 mmol of Tris-HCl, pH 7.5; 5 mmol of CaCl2; 100 mmol of NaCl; 1 mmol of ZnCl2; 0.2 g of Brij®-35; 2.5 mL of Triton X-100; and 0.02 g of NaN3) (32). Zymograms were incubated in the presence of 5 mmol/L EDTA and 2 mmol/L 1,10-phenanthroline for inhibition studies (31); activation of zymogens was achieved with 2 mmol/L p-aminophenyl-mercuric acetate (31). Staining was performed in Coomassie brilliant blue R-250 (2 g/L), and gels were destained appropriately (32). Aliquots containing 150 μg of total protein were used.

Gelatinolytic bands were measured densitometrically with an image analyzer (Cybernetics) (33). Gelatinase calibrators were prepared by diluting healthy capillary blood with 15 volumes of nonreducing sample buffer (containing, per liter, 62.5 mmol of Tris-HCl, pH 6.8; 350 mL of glycerol; 25 mL of Triton X-100; 40 g of sodium dodecyl sulfate; 0.2 g of Brij-35; 0.02 g of NaN3; and 0.1 g of bromphenol blue) (34).

To investigate the ability of breast cells to produce and/or accumulate gelatinases and to evaluate their diagnostically accuracy, we studied molecular forms of MMP-2 and MMP-9 in NAF subtypes, evaluating their concentrations by immunoassay and their isoform distribution by gelatin zymography (31).

The present work was carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

The linearity and interference studies revealed squared correlation coefficients (r2) between MMP concentrations and dilution of 0.97 and 0.94 for MMP-2 and MMP-9, respectively, suggesting that the NAF matrix did not affect the performance of immunoassays.

The mean (SE) recoveries of purified MMPs added to NAFs were 92 (5)% and 95 (7)% for MMP-2 and MMP-9, respectively. Intra- and interassay CVs were 5% and 12% for MMP-2 and MMP-9, respectively.

Using a previous classification (26, 28, 30), we subdivided the NAFs as type I (23 healthy and 45 BBD-affected women) and type II (12 BC patients and 5 women originally diagnosed for BBD who subsequently developed BC).

As shown in Table 1, the mean MMP-2 in type I NAFs (healthy women and women with BBD; n = 68) was significantly lower than that in type II NAFs from BC patients (n = 17; P < 0.005). We also found a significant difference between BBD and BC patients (244 (23) μg/L in BBD (n = 45) vs 336 (26) μg/L in BC (n = 17); P < 0.005).

MMP-concentrations were higher in NAFs from women with BBD than in healthy controls [232 (27) μg/L vs 166 (12) μg/L; P < 0.01] and were the highest in BC patients (P < 0.01 vs controls and P < 0.05 vs BBD, BBD patients).
respectively). Type I NAFs contained significantly lower MMP-9 than type II NAFs ($P < 0.001$).

In NAFs ($n = 5$) from patients diagnosed as BBD who subsequently developed BC, the mean (SE) concentrations were $291 \pm 26 \mu g/L$ and $365 \pm 32 \mu g/L$ for MMP-2 and MMP-9, respectively ($P < 0.001$ vs controls and $P < 0.01$ vs BBD patients).

Gelatin zymography detected all blood circulating gelatinases [72-kDa fibroblast-derived proMMP-2 and 92-, 130-, and 225-kDa neutrophil-derived proMMP-9 (33, 34)] biochemically and immunologically demonstrated to be MMP-2 and MMP-9 (Fig. 1A).

Zymography of type I NAFs ($n = 68$) revealed four gelatinolytic bands, with molecular masses similar to those obtained for gelatinases from capillary blood, that were biochemically inhibited by EDTA and 1,10-phenanthroline (Fig. 1B), were demonstrated immunologically to be MMP-2 and -9, and accumulated in NAFs as zymogens (see Fig. 1, panels a and b, in the Data Supplement that consists of 1548 Technical Briefs).

### Table 1. Mean (SE) MMP concentrations in plasma and NAF subtype samples.

<table>
<thead>
<tr>
<th></th>
<th>Heparin plasma (n = 85)</th>
<th>Type I NAFs (n = 68)</th>
<th>Type II NAFs (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2, $\mu g/L$</td>
<td>119.3 (9.6)</td>
<td>202.8 (18.4)</td>
<td>336.0 (26.2)</td>
</tr>
<tr>
<td>MMP-9, $\mu g/L$</td>
<td>19.7 (1.4)</td>
<td>119.1 (17.6)</td>
<td>498.0 (95.2)</td>
</tr>
</tbody>
</table>

*° Type I NAFs vs type II NAFs: $^*P < 0.005$; $^{**}P < 0.001$.

Fig. 1. Gelatin zymograms of MMPs from capillary whole blood and NAF subtypes.

Samples (150 $\mu g$ of total protein) were analyzed on 7.5% gels containing 2.0 g/L gelatin 90 Bloom. Molecular masses (expressed in kDa) are indicated. (A), Western blot analysis of MMPs present in capillary whole blood (lane Std), using monoclonal anti-MMP-9 (lane 1) and anti-MMP-2 (lane 2) antibodies. (B), MMP isoforms detected in four representative type I NAFs (lanes 1 and 4, NAF from control, healthy women; lanes 2 and 3, NAF from patients affected by BBD). Cation-dependent gelatinolytic activity was tested by incubation of type I and II NAFs with EDTA and 1,10-phenanthroline (lanes 5 and 6, respectively). (C), MMP isoforms in four representative samples of type II NAFs. Lanes 1 and 3, cancer-bearing patients; lanes 2 and 4, patients originally diagnosed for BBD who subsequently developed BC. MMP inhibition by cation chelators is also shown (lanes 5 and 6).
accompanied the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue10/).
The 92-kDa gelatinase B appears to be the constitutive MMP form.

Zymograms obtained from type II NAFs (n = 17) showed mainly two bands (92 and 72 kDa) with gelatinolytic activity that was inhibited by cation chelators (Fig. 1C), with gelatinase A as the constitutive form. p-Aminophenyl-mercuric acetate activation and Western blot analyses demonstrated that the gelatinases in type II NAFs were the proforms and activated forms of MMP-2 and -9 (Fig. 1, panels c and d, in the online Data Supplement).

NAFs from BBD patients who subsequently developed BC showed additional gelatinolytic bands (activated forms of both gelatinase A and B; Fig. 1C, lanes 2 and 4). Densitometrically, the gelatinase concentrations appeared to be two- to fourfold higher in type II NAFs than in type I NAFs (P < 0.01).

Comparing gelatinases in NAFs from the BBD or BC breasts of patients with the contralateral healthy breasts (n = 6), we found that the higher MMP concentrations and peculiar zymograms of type II NAFs may better represent a local process of higher biosynthetic activity.

Residual gelatinolytic activity was ~70% after 30 min at 55 °C and ~20% at 65 °C; MMP forms were not altered. In this study, we found that NAFs contain MMP-2 and -9 and that both are significantly higher in type II NAFs collected from cancer-bearing patients than in type I from healthy and BBD-affected women; moreover, zymography revealed gelatinase profiles related to the pathologic state. Type I NAFs (healthy and BBD women) showed the same MMP forms circulating in blood, suggesting a mechanism of passive plasma filtration. In type II NAFs (cancer-bearing patients), we detected mainly two strongly expressed MMPs, the possible source being an active synthesis by breast cells and accumulation in NAFs in both the zymogenic and activated forms.

Our results (higher concentrations of gelatinase A in type I NAFs from healthy and BBD tissue) are in agreement with findings of MMP production/secretion in healthy breast tissue (3, 4, 6, 20, 22). Although the physiologic roles of MMPs in the breast are not completely understood, our results may be related to the remodeling functions of breast in toto (3, 22).

Related to MMPs synthesis/secretion by neoplastic and stromal cells, extensive ECM remodeling in breast tissue occurs during cancer initiation and progression (3, 5, 7–9, 13, 14, 19). Our findings of strong expression for the proforms and active forms of MMP-2 and -9 in type II NAFs are in agreement with the involvement of cancer cells in enhanced gelatinolytic activity during neoplastic evolution (4, 11, 19).

Although the origin of NAF gelatinases is unknown, we suggest that accumulation of pro- and activated forms of MMPs in type II NAFs may derive from the highly metabolizing apocrine cells (26, 29, 37), representing a potential cancer biomarker with diagnostic accuracy. The production and/or secretion by breast cells of several proteolytic enzymes (26, 29, 37) and biologically active targets for MMP activity (3), in association with a lack of physiologic control of the NAF secretion/reabsorption mechanism (23) and with prolonged exposure to several biologically active substances through the autocrine/paracrine mechanism (27), could, with age, make the biosynthetically active apocrine NAF cells prone to pre-malignant transformation (25–27). Biologic evaluation of the balance between MMP activity and MMP inhibitors (21, 38) may clarify the biological mechanisms of gelatinases during early neoplastic transformation (3, 4).

References