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Letter to the Editor

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Validation of the enhanced liver fibrosis (ELF)-test in heparinized and EDTA plasma for use in reflex testing algorithms for metabolic dysfunction-associated steatotic liver disease (MASLD)

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To the Editor,

Metabolic dysfunction-associated steatotic liver disease (MASLD) is rapidly becoming the most common liver disease worldwide, currently effecting around a third of the adult population [1]. Diagnostic algorithms that use non-invasive tests (NITs) to stage MASLD and allow for the non-invasive and early detection of advanced stages of MASLD fibrosis are currently being developed and implemented [2–4]. An often-proposed strategy is the use of two or more sequential NITs to accurately and cost-effectively assess the risk of underlying fibrosis. Commonly, these combinations start with the FIB-4 score, a low-cost and easy-to-use NIT requiring age, AST, ALT and platelets, followed by a more expensive, more robust test such as the enhanced liver fibrosis (ELF)-test. Reflex testing with such combinations would enhance the diagnostic process for fibrotic MASLD. Yet, whereas transaminases are commonly measured in heparinized plasma and platelets are measured in EDTA plasma, the ELF-test has only been validated for use in serum. Therefore, we aimed to validate the ELF-test in both heparinized plasma and EDTA plasma.

To this end, we utilized the Nijmegen-Leiden-Amsterdam (NLA)2 study, consisting of patients at risk of MASLD from primary, secondary and tertiary care who were screened with NITs (i.e., FIB-4 score and vibration controlled transient elastography (VCTE)), as the derivation cohort. Blood samples consisting of serum, heparinized plasma and EDTA plasma were collected and stored in a

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designated biobank. The validation cohorts consist of a selection of the NAFLD In the healthy Life in an urban sEtting (NILE) cohort, representing a primary care population at risk of MASLD, and the Amsterdam NAFLD-NASH cohort (ANCHOR), representing a secondary and tertiary care population with histologically characterized MASLD [5, 6]. The three ELF proteins (hyaluronic acid (HA), PIIINP and TIMP-1) were analyzed separately using ELF-test kits provided by Siemens™ according to manufactures' instructions using the Atellica IM analyser (Siemens Heathineers) [7]. All samples were analyzed simultaneously at the endocrinology laboratory at the Amsterdam UMC, location AMC.

The NLA2 derivation cohort consisted of 144 participants with a mean age of 57.9 (12.3) years (Supplementary Table 1). 63.9 % of participants were women and 55.6 % of participants had type 2 diabetes mellitus. Mean BMI was 31.5 (5.5) kg/m^2, 90.2 % had BMI ≥25 kg/m^2 and 57.6 % had BMI ≥30 kg/m^2. 61.1 % of participants had controlled attenuation parameter (CAP) ≥290 dB/m and 22.9 % had a liver stiffness measurement (LSM) ≥8.2 kPa, suggesting the presence of ≥S3 steatosis and ≥F3 fibrosis [8]. Mean ELF-score in heparinized plasma was comparable to that in serum (9.06 (0.79) vs. 9.06 (0.76) (p=0.999)), while mean ELF-score in EDTA plasma was lower than ELF-score in serum (8.69 (0.78) vs. 9.06 (0.76) (p<0.001)) (Supplementary Table 2). Correlation coefficients between ELF-score in serum vs. heparinized plasma and EDTA plasma were 0.974 and 0.966 (p for both <0.001), respectively. Regarding the three individual components of ELF, median HA in heparinized plasma and EDTA plasma were not significantly different from median HA in serum (p=0.680 and p=0.613, respectively), but median PIIINP and TIMP-1 were significantly different (all p<0.001) (Supplementary Table 2). Given the differences in levels of the individual ELF proteins, correction factors were calculated using Passing–Bablok regression analyses and new formulas were designed using the original formula for the ELF-score as reference:

Heparinized plasma

$$\begin{aligned} ELF = {} & 2.278 + 0.851 \cdot (\ln((HA - 0.825)/0.982)) \\ & + 0.751 \cdot (\ln((PIIINP + 0.607)/1.407)) \\ & + 0.394 \cdot (\ln((TIMP1 + 13.080)/0.638)) \end{aligned}$$

EDTA plasma

$$\begin{aligned} ELF = {} & 2.278 + 0.851 \cdot (\ln((HA - 2.494)/0.950)) \\ & + 0.751 \cdot (\ln((PIIINP + 0.757)/0.881)) \\ & + 0.394 \cdot (\ln((TIMP1 + 33.909)/0.743)) \end{aligned}$$

Using the corrected formula for ELF-test in heparinized plasma, mean ELF-score was 9.07 (0.79) compared to 9.06 (0.76) in serum (p=0.983). Pearson's correlation resulted in a correlation coefficient of 0.975 (p<0.001). Mean ELF-score using the corrected formula in EDTA plasma was also 9.07 (0.80) (p=0.974) and the correlation coefficient with ELF-score in serum was 0.969 (p<0.001).

In the NILE cohort, mean ELF-score in EDTA plasma using the unadjusted formula was significantly lower than mean ELF-score in serum (8.85 (0.82) vs. 9.26 (0.79) (p<0.001)). Application of the corrected formula resulted in a mean ELF-score in EDTA plasma comparable to that in serum (9.23 (0.82) vs. 9.26 (0.79) (p=0.823)) (Supplementary Table 2). Mean ELF-score in heparinized plasma was comparable to serum using the unadjusted and the corrected formulas. When comparing agreement between ELF-test conducted in heparinized plasma and serum, Cohen's kappa increased from 0.84 to 0.87 when using the corrected formula compared to the unadjusted formula. When using ELF-test conducted in EDTA plasma, Cohen's kappa increased from 0.68 to 0.90.

In the ANCHOR cohort, mean ELF-score in heparinized plasma and EDTA plasma using the unadjusted formulas was comparable to mean ELF-score in serum (9.17 (0.85) vs. 8.91 (0.86) (p=0.092), and 8.67 (0.86) vs. 8.91 (0.86) (p=0.133), respectively). When using the corrected formulas, the mean ELF-score remained comparable between serum and heparinized plasma and EDTA plasma (9.16 (0.84) vs. 8.91 (0.86) (p=0.100), and 9.03 (0.87) vs. 8.91 (0.86) (p=0.411), respectively) (Supplementary Table 2). Figure 1 shows Bland-Altman plots of serum and heparinized plasma and serum and EDTA plasma using the unadjusted and the corrected formulas in the validation cohorts. Sensitivity of the ELF-test for the detection of ≥F3 fibrosis was higher using the corrected formulas for heparinized plasma (0.53 (0.29, 0.77) vs. 0.47 (0.23, 0.71)) and EDTA plasma (0.53 (0.29, 0.77) vs. 0.29 (0.08, 0.51)) compared to the unadjusted formulas. The AUC of ELF-test with the predefined cut-off of 9.8 to detect ≥F3 fibrosis was higher for the corrected formulas for heparinized plasma (0.71 (0.58, 0.84) vs. 0.68 (0.55, 0.81)) and EDTA plasma (0.70 (0.56, 0.83) vs. 0.62 (0.51, 0.74)) compared to the unadjusted formulas (Supplementary Table 3).

To explore the concept of reflex testing, the Camden & Islington algorithm [9] – in which an ELF-test follows an intermediate FIB-4 score (1.30–3.25) – was retrospectively applied in the ANCHOR cohort. 19 participants (31.7 %) had an intermediate FIB-4 score and would thus be provided an ELF-test for which the manufacturer's cut-off of 9.8 was used (Supplementary Figure 1). One participants was omitted from analyses due to insufficient data to calculate the FIB-4 score. The sensitivity and specificity of this reflex testing algorithm using ELF-test performed in serum were 0.38 (0.14, 0.61) and 0.93 (0.86, 1.00), yielding an AUC of 0.70 (0.55, 0.84). Using heparinized plasma or EDTA plasma with the unadjusted formulas yielded AUCs of 0.69 (0.54, 0.83) and

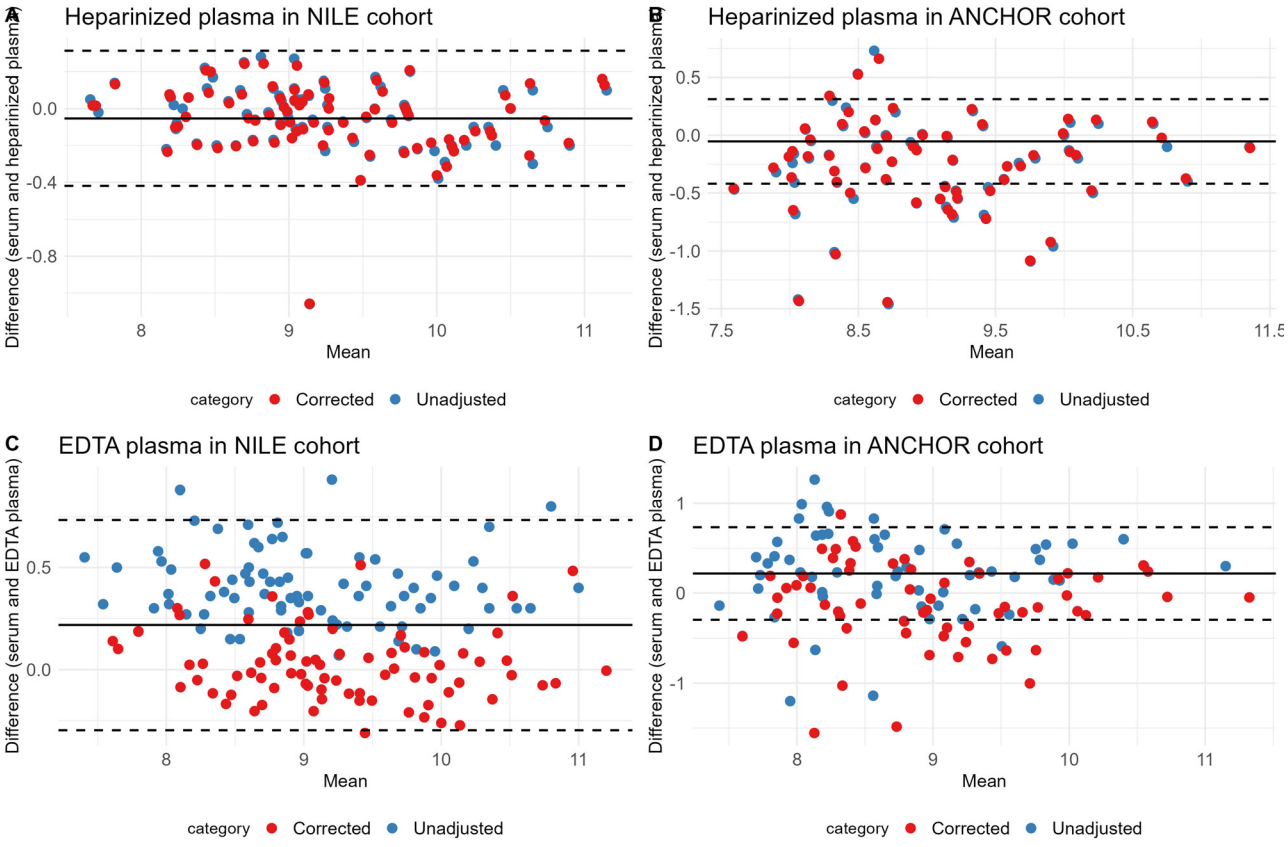

Figure 1: Bland–Altman plots of ELF-score in serum and heparinized plasma using the unadjusted and the corrected ELF-score formulas in the NILE (A) and ANCHOR validation cohorts (B) and ELF-score in serum and EDTA plasma using the unadjusted and the corrected ELF-score formulas in the NILE (C) and ANCHOR validation cohorts (D).

0.67 (0.52, 0.82), and application of the corrected formulas yielded AUCs of 0.70 (0.56, 0.85) and 0.69 (0.55, 0.84), respectively (Supplementary Table 4).

Taken together, here we demonstrate that ELF-test performed in heparinized plasma and EDTA plasma has comparable performance to that of ELF-test performed in serum when using correction factors. The usefulness of the corrected ELF-test formulas is demonstrated in separate validation cohorts. This allows for the application of reflex testing algorithms with the ELF-test as a second-tiered test. Sensitivity and AUC for the detection of ≥F3 fibrosis increased when performing the ELF-test in heparinized plasma and EDTA plasma using the corrected compared to the unadjusted formulas. Interestingly, AUCs, including that of ELF-test performed in serum, are considerably lower than previously reported in a meta-analysis by Vali et al. who reported an AUC of 0.83 (0.71, 0.90) [10]. There are some study limitations including the relatively small sample size of the histologically characterized validation cohort and unequal distribution of MASLD-subtypes. Nevertheless, our study has strong clinical utility: the validation of ELF-test in heparinized plasma and EDTA plasma allows for direct applicability of the ELF-test in reflex testing algorithms for fibrotic MASLD.

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Research ethics: All studies were approved by the Medical Ethical Committee of the Amsterdam UMC, Amsterdam, The Netherlands, and the study was conducted in accordance with the declaration of Helsinki (as revised in 2013).

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

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Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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