ing to replacement of the three C-terminal amino acids, α139–141, by Asn139-Thr-Val-Lys-Leu-Glu-Pro-Arg146 together with partial deamidation of Asn139 (40%) to Asp139 (60%). The patient was heterozygous for the variant, which was present at 18% of total α-chains.

The previously reported cases of Hb Wayne were identified by biochemical methods. In electrospray ionization/MS, ions are formed from intact polypeptides by adding one or more protons (H) (5). MS measures the mass to charge ratio (m/z) of these ions. Because the number of charges (n) is known, the molecular mass (Mr) in daltons (Da) is deduced using Mr = n(m/z - H), and software converts the original m/z data into a mass spectrum as in Fig. 1 of the data supplement (available with the online version of this Technical Brief at http://www.clinchem.org/content/vol48/issue12/). In effect, MS/MS combines the purification and sequencing of polypeptide mixtures, previously undertaken by the use of several procedures into one operation without chromatography. The technique uses an instrument consisting essentially of two mass spectrometers in series. The first spectrometer purifies the polypeptide mixture by selecting ions with a specific m/z. These ions are then fragmented by collisions with a gas in a cell situated between the two spectrometers. The resulting fragment ions are then analyzed according to their m/z in the second spectrometer to give sequence information. Mainly, fragment ions result from cleavages between the amino acid residues in the polypeptide chain. The skill in MS lies in identifying diagnostic peaks in complex spectra with the aid of computer programs. The complete MS analysis of the variant Hb was carried out and results were returned to the referring laboratory within 4 working days, a substantial reduction compared with biochemical techniques.

This is the third abnormal Hb we have reported that has been analyzed by this technique (6–8), and we recommend it as a rapid method for identification of unknown variants, although we caution that interpretation requires considerable expertise.

References

Different Kinetics of Bone Markers in Normal and Delayed Fracture Healing of Long Bones, Markus Herrmann1, Daniela Klitscher1, Thomas Georg2, Johannes Frank3, Ingo Marzi3, and Wolfgang Hermann1

We investigated 14 patients with a traumatic crural (n = 13) or femoral (n = 1) shaft fracture (Table 1). All patients underwent operative therapy and were monitored for 1 year. No patient suffered from diseases or had been administered medication known to interfere with bone metabolism. Blood samples for measurement of OC, bone ALP, and β-CTx were taken within 24 h of fracture and after 7, 14, 28, 42, 60, 90, 180, and 365 days. Serum was separated from whole blood and stored at −20°C until measurement. We also evaluated a clinical score of fracture healing. At the end of the study, patients were classified as having normal or delayed fracture healing. The rate of healing was considered normal if the fracture healed completely after 6 months (score 4) and score 2 was reached on day 60. Otherwise, fracture healing was classified as delayed.

The clinical score was defined as follows: (0), no weight bearing, strong inflammation, pain at rest; (1), mobilization with crutches, partial weight bearing (less than
The patients with delayed fracture healing showed a significant lag of the clinical score between the 42nd and 180th days. At 2 months, this difference was most pronounced (Fig. 1). At the end of the study, there was no significant difference between the two groups.

OC increased during fracture healing [Fig. 1 and Data Supplement Table (available with the online version of this Technical Brief at http://www.clinchem.org/content/vol48/issue12/)]. In patients with delayed healing, OC began to increase 1 month later than in patients with normally healing fractures (28th vs 60th day). Moreover, the OC peak was delayed by ~1 month (60th vs 90th day). Afterward, OC returned to baseline in both groups. d(OC) was significantly higher in patients with normal fracture healing on day 42 ($P = 0.034$) and on a 10% level also on day 28 ($P = 0.090$). OC is synthesized as a noncollagenous matrix protein by osteoblasts during callus formation. The delayed increase of OC in patients with delayed healing may reflect the fact that fracture healing depends heavily on sufficient callus formation between the first and fourth months after fracture (11).

Bone ALP was less predictive than OC (Fig. 1 and Data Supplement Table). Bone ALP increased significantly between the 28th and 42nd days in normal fracture healing and peaked between the 42nd and 90th days. In patients with delayed healing, bone ALP at baseline was significantly higher and tended to increase throughout the study period. d(bone ALP) seemed to be smaller in delayed-healing than in normal-healing fractures between the 42nd and 90th days. $\beta$-CTx did not help to differentiate the two groups.

Our data indicate a significant lag in increases of OC and possibly also bone ALP in patients with delayed fracture healing during the first 2 months. In normal healing, there is a significant increase of OC above baseline at the 42nd day, whereas there is none in delayed healing. Because plain radiography cannot distinguish between normal and delayed healing at this time, longitudinal measurement of OC and bone ALP may be helpful for earlier identification of patients at risk. Additionally, OC provides information about osteoblastic activation and callus formation while x-rays reflect only the calcified tissue in this region.

Only a few studies have attempted to identify differences in the kinetics of OC and bone ALP in patients with delayed fracture healing (12–15). All of these studies considered only mean values of OC and bone ALP. Given that both markers show large interindividual variations,
possible effects can be lost by the use of mean values. We therefore calculated intraindividual differences between each time of measurement and the corresponding baseline value.

Emami et al. (13) found increases of OC and bone ALP during fracture healing but no differences in the kinetics. Viewing the data provided in that report, however, we see an apparent lag in delayed fracture healing within the first 2 months for both markers. Possibly the calculation of intraindividual differences would have elucidated such a delay. The higher bone ALP values in normal-healing fractures during the first 3 months are in contrast to our results. This discrepancy may reflect the different methods used for measurement of bone ALP. Emami et al. (13) measured protein concentrations, whereas our method detected bone ALP activity, which better represents osteoblastic activity.

Oni et al. (12) reported lower OC concentrations in delayed fracture healing, which is not consistent with our results, but the comparability of these studies is compromised by the different study designs, e.g., conservative vs operative fracture treatment and different methods for the measurement of OC. Their test detected only the entire OC molecule and not the more stable cleavage product N-MID fragment. The summation of OC and N-MID fragment may provide a better indicator of osteoblastic activity and reduce interindividual variations. Furthermore, Oni et al. (12) did not calculate intraindividual differences. Nevertheless, they reported a slight lag of the bone ALP increase in delayed fracture healing, which is consistent with our results. All other studies considering these markers, including our earlier publication, did not distinguish normal and delayed fracture healing (16–20).

Given that all studies to date, including our own, had only small numbers of participants with delayed fracture healing, we tried to pool data from the literature and our data. Because of methodologic differences or insufficient data presentation in the publications, we could pool only the OC data from Emami et al. (13) and our present study. Together we had 11 patients with delayed and 33 with normal fracture healing. The studies measured OC with the same method. Baseline OC was similar in both groups (16.1 μg/L). After 4 weeks, OC remained almost unchanged in delayed healing (16.1 ± 4.5 vs 17.5 ± 5.0 μg/L; P = 0.48), whereas in normal healing there was a significant increase (16.1 ± 6.7 vs 24.2 ± 11.3 μg/L; P < 0.001). Unfortunately, Emami et al. gave no data for week 6. This pooled analysis supports our finding that there is a significant lag of the OC increase in patients with delayed fracture healing that can be detected by sequential OC measurements during the first 2 months.

The present study is a pilot study that has major limitations in the small number of patients. Therefore, the emphasis of the present data lies more on the descriptive presentation of the data. Nevertheless, we performed a statistical exploration to give an indication of the possible
relevance of the observed effects. We conclude that the sequential measurement of OC and possibly also of bone ALP during the first 2 months after fracture might be used to predict delayed fracture healing. Previous studies have been very limited, and because of methodologic problems, they are only partly comparable with our study. Further studies are needed.

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References


Use of Methyl Malondialdehyde as an Internal Standard for Malondialdehyde Detection: Validation by Isotope-Dilution Gas Chromatography–Mass Spectrometry, Giuliana Cighetti,1 Pietro Allevi,2 Luigi Anastasia,3 Luana Bortone,1 and Rita Paroni2 (1 Department of Medical Chemistry, Biochemistry and Biotechnology, School of Medicine, University of Milan, Via Saldini 50, 20133 Milan, Italy; 2 Dipartimento di Medicina, Chirurgia e Odontoiatria, Università di Milano, H. San Paolo, Via Di Rudini 8, 20142 Milan, Italy; 3 Department of Chemistry, Purdue University, West Lafayette, IN 47907-1393; 4 IRCCS H San Raffaele, Via Olgettina 60, 20132 Milan, Italy; * author for correspondence: fax 39-2-50316040, e-mail giuliana.cighetti@unimi.it)

Malondialdehyde (MDA), a compound derived from lipid peroxidation and from eicosanoid biosynthesis, exists in biological matrices both in the free form and bound to SH and/or NH2 groups of various biomolecules (1). Although other compounds (isoprostanes) have been proposed as more reliable indicators of oxidative damage (2), MDA is still widely used in clinical chemistry laboratories to monitor oxidative stress (3). Several methods have been developed to evaluate MDA in biological samples (1,4), but the different analytical conditions used and the lack of a suitable internal standard have led to large discrepancies in measurements even at physiologic MDA concentrations in human plasma (5).

We (6) recently reported a “reference method” for free and total plasma MDA quantification, as the phenylpyruvate derivative, by isotope-dilution gas chromatography–mass spectrometry (ID-GC-MS) with deuterated MDA (d2-MDA) as internal standard. This method, used for clinical MDA detection (7–9), offers the possibility of validating other proposed internal standards that differ from MDA in structure, stability, and reactivity. Unfortunately, the major limitations of d2-MDA include its difficult synthesis (10) and that it is detectable only by GC-MS, a method not always available in clinical laboratories. A compound that appears to be more suitable as an internal standard is methyl malondialdehyde (MMDA) because it is structurally close to MDA, is absent from biological matrices, is easily obtainable from a commercial compound, and is detectable by common methods such as HPLC, GC, and capillary electrophoresis.

MMDA was first evaluated as an internal standard for MDA determinations by Bull and Marnett (11), who unfortunately experienced difficulties in resolving the underivatized MDA and MMDA by HPLC. Recently, Claeson et al. (12) reported the use of MMDA as an internal standard for measurement of MDA in rat brain by capillary electrophoresis, thus avoiding the derivatization step. We successfully adapted this method for detection of MDA in rat liver microsomes and human plasma (13) and tested it against the ID-GC-MS method.

Claeson et al. (12) also proposed the use of MMDA for free MDA detection by GC with prior derivatization with pentafluorophenylhydrazine. However, some problems remained unresolved, such as the performance of MMDA