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High-Throughput Simultaneous Analysis of RNA, Protein, and Lipid Biomarkers in Heterogeneous Tissue Samples

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BACKGROUND: With expanding biomarker discovery efforts and increasing costs of drug development, it is critical to maximize the value of mass-limited clinical samples. The main limitation of available methods is the inability to isolate and analyze, from a single sample, molecules requiring incompatible extraction methods. Thus, we developed a novel semiautomated method for tissue processing and tissue milling and division (TMAD).

METHODS: We used a SilverHawk atherectomy catheter to collect atherosclerotic plaques from patients requiring peripheral atherectomy. Tissue preservation by flash freezing was compared with immersion in RNAlater, and tissue grinding by traditional mortar and pestle was compared with TMAD. Comparators were protein, RNA, and lipid yield and quality. Reproducibility of analyte yield from aliquots of the same tissue sample processed by TMAD was also measured.

RESULTS: The quantity and quality of biomarkers extracted from tissue prepared by TMAD was at least as good as that extracted from tissue stored and prepared by traditional means. TMAD enabled parallel analysis of gene expression (quantitative reverse-transcription PCR, microarray), protein composition (ELISA), and lipid content (biochemical assay) from as little as 20 mg of tissue. The mean correlation was $r = 0.97$ in molecular composition (RNA, protein, or lipid) between aliquots of individual samples generated by TMAD. We also demonstrated that it is feasible to use TMAD in a large-scale clinical study setting.

CONCLUSIONS: The TMAD methodology described here enables semiautomated, high-throughput sampling of small amounts of heterogeneous tissue specimens by multiple analytical techniques with generally improved quality of recovered biomolecules.

The implementation of efficient methods for processing and analysis of biological samples is an important component of the execution of clinical trials. The main limitation of available methods is the inability to isolate and analyze, from a single specimen, molecules requiring incompatible extraction methods.

Because most tissue samples are compositionally heterogeneous, e.g., atherosclerotic plaque *(1)* (Fig. 1) and tumor tissue *(2)*, extraction and analysis of biomarkers from different fragments can lead to misleading results. Therefore, the development of a universal

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[3] Nonstandard abbreviations: TMAD, tissue milling and division; TT1, TissueTube 1, TMAD, tissue milling and division; TLDA, TaqMan low-density array; RIN, RNA integrity number; IL, interleukin; MIP-1β, macrophage inflammatory protein-1β; MCP-1, monocyte chemotactic protein-1; RANTES, regulated on activation normal T-cell expressed and secreted; CRP, C-reactive protein; TNFRII, tumor necrosis factor receptor-II; ICAM-1, intercellular adhesion molecule-1; MPO, myeloperoxidase; PAD, peripheral artery disease.

Human genes: *PLA2G7*, phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma); *DUSP1*, dual specificity phosphatase 1; *CCL2*, chemokine (C-C motif) ligand 2; *ALOX5AP*, arachidonate 5-lipoxygenase-activating protein; *AHCY*, adenosylhomocysteinase; *PTGS2*, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); *HMOX1*, heme oxygenase (decycling) 1; *MPO*, myeloperoxidase; *CCR1*, chemokine (C-C motif) receptor 1; *CCL7*, chemokine (C-C motif) ligand 7; *4342379–18S*, *VCAM1*, vascular cell adhesion molecule 1; *CD5L*, CD5 molecule-like; *FOS*, FBJ murine osteosarcoma viral oncogene homolog; *ABCA1*, ATP-binding cassette, sub-family A (ABC1), member 1; *TNF*, tumor necrosis factor; *HCAR3*, hydroxycarboxylic acid receptor 3; *PTAFR*, platelet-activating factor receptor; *CCL3*, chemokine (C-C motif) ligand 3; *MMP9*, matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase); *MMP1*, matrix metallopeptidase 1 (interstitial collagenase); *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *CXCL2*, chemokine (C-X-C motif) ligand 2; *IL8*, interleukin 8; *ALOX5*, arachidonate 5-lipoxygenase; *CBS*, cystathionine-beta-synthase; *CYSLTR2*, cysteinyl leukotriene receptor 2; *CD163*, CD163 molecule; *EGR2*, early growth response 2; *CCL5*, chemokine (C-C motif) ligand 5; *LTB4R*, leukotriene B4 receptor; *ADAMTS1*, ADAM metallopeptidase with thrombospondin type 1 motif, 1; *CTSL1*, cathepsin L1; *ACTB*, actin, beta; *IL6*, interleukin 6 (interferon, beta 2); *CRP*, C-reactive protein, pentraxin-related; *NR4A1*, nuclear receptor subfamily 4, group A, member 1; *IL1A*, interleukin 1, alpha; *PPIA*, peptidylprolyl isomerase A (cyclophilin A); *PCNA*, proliferating cell nuclear antigen; *SPP1*, secreted phosphoprotein 1; *SELE*, selectin E; *SCARB1*, scavenger receptor class B, member 1; *CYSLTR1*, cysteinyl leukotriene receptor 1; *PLA2G10*, phospholipase A2, group X; *NOS2*, nitric oxide synthase 2, inducible; *ITGAX*, integrin, alpha X (complement component 3 receptor 4 subunit); *ICAM1*, intercellular adhesion molecule 1.

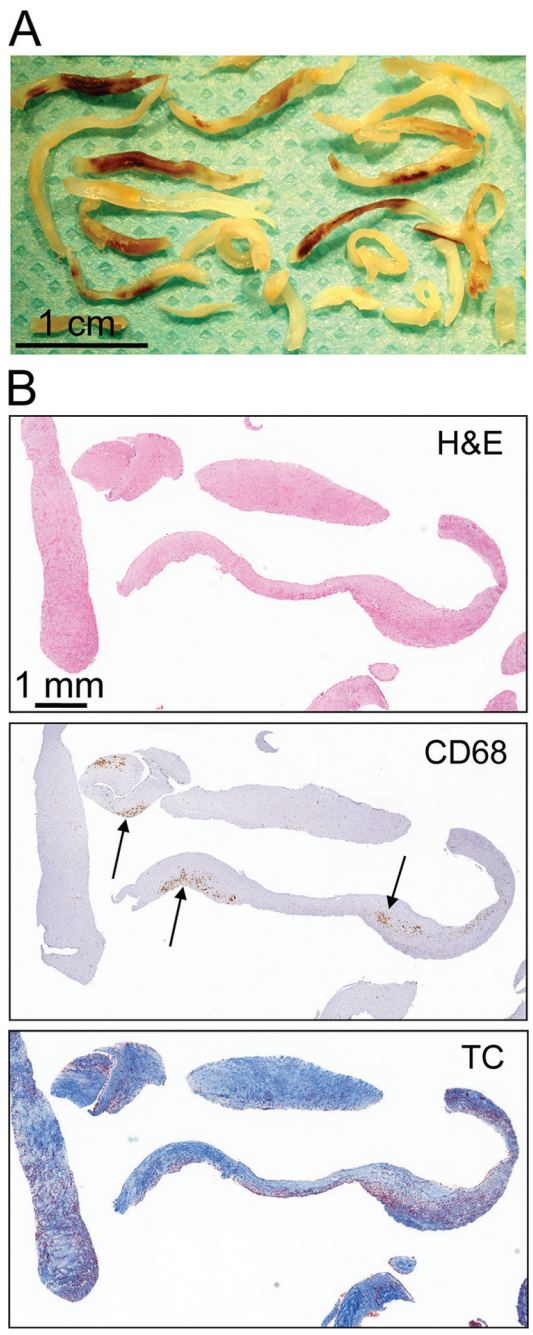


Fig. 1. Excised peripheral plaque is both physically and compositionally heterogeneous.

(A), A set of excised peripheral plaque tissue samples from a single procedure with the SilverHawk device. (B), Histological analysis of adjacent longitudinal sections (5 μm) of plaque stained with hematoxylin and eosin (H&E) showing overall tissue architecture, macrophage marker (CD68) with arrows indicating macrophage localization, and Gomori's trichrome (TC), identifying smooth muscle cell content.

method for dividing a clinical specimen into compositionally identical aliquots is critically important.

We developed and validated a semiautomated tissue-splitting technique, tissue milling and division (TMAD),[3] that processes tissue into fine homogeneous powder that can be divided into identical aliquots for differential extraction. In addition, we demonstrated that preservation of atherosclerotic plaque in RNAlater is compatible with quantitative analysis of mRNA, proteins, and lipids in tissue extracts.

Materials and Methods

TISSUE COLLECTION METHODS

Plaque tissue was excised with a SilverHawk atherectomy catheter (ev3), generating a set of plaque tissue samples from each lesion (Fig. 1A). After removal from the SilverHawk device the tissue was rinsed with 4 °C 0.9% NaCl saline.

Flash-frozen preservation method. Each set of samples was put into a 2-mL cryovial and frozen in liquid nitrogen within 5 min of excision. Samples were shipped on dry ice and stored at −80 °C.

RNAlater preservation method. Each set of samples was put into a 5-mL cryovial containing 4.5 mL RNAlater (Ambion) within 5 min of excision. Tissue was stored in RNAlater at 4 °C for a minimum of 12 h, shipped on frozen gel packs, and stored at −20 °C.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

Plaque fragments were fixed in 10% neutral buffered formalin, processed through graded alcohols and a clearing agent, and embedded in paraffin blocks. Tissue sections (5-μm thick) were stained with hematoxylin and eosin, Gomori's trichrome, and elastic Van Gieson stains. For detection of macrophages, sections were incubated with mouse anti–human CD68 antibody (clone PG-M1; Dako) followed by horseradish peroxidase–labeled antimouse antibody (Dako Envision System), and then visualized with 3,3′-diaminobenzidine.

TISSUE-PROCESSING METHODS

Standard method. We processed frozen and RNAlater tissue samples by impact pulverization using Covaris Cryo-Prep (Covaris). Briefly, the tissue was placed in the lower portion of the TissueTube 1 (TT1) bag and submerged in liquid nitrogen for approximately 5 s. The bag was then placed into the instrument and pulverized. Usually 2 rounds of freezing/pulverization were necessary to obtain tissue pieces <2 mm in size. Tissue fragments were then frozen again in liquid ni-

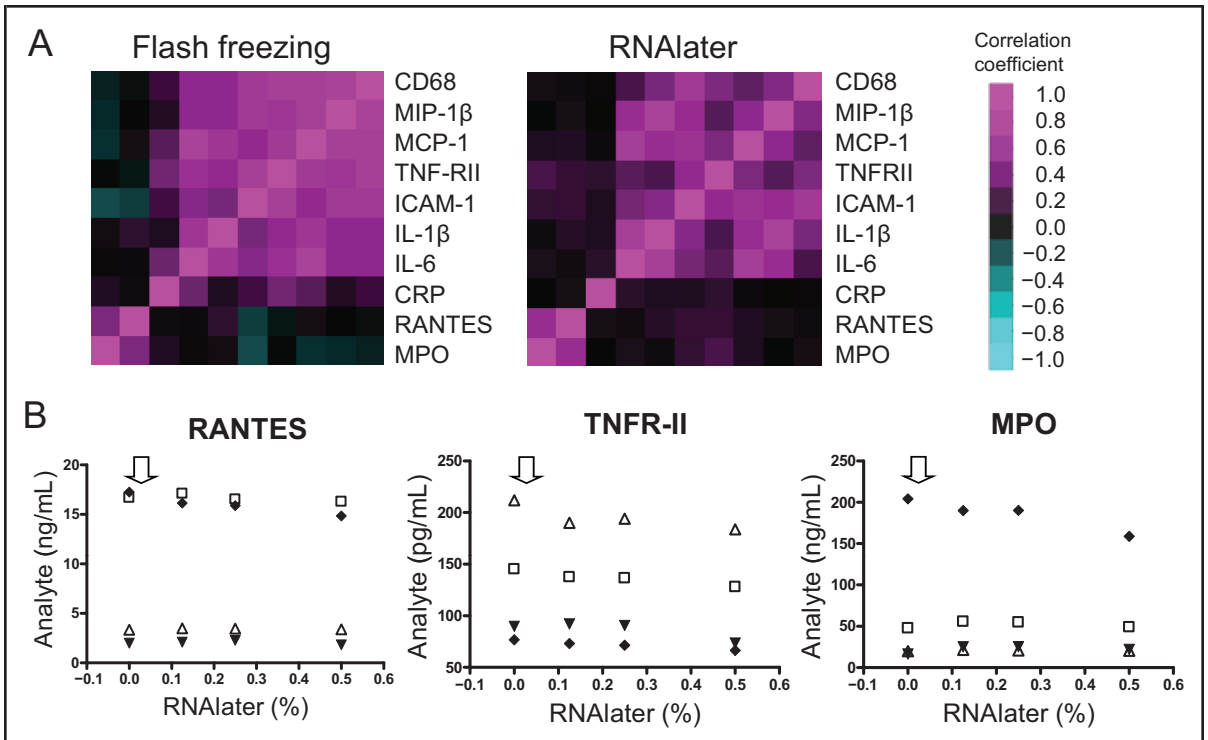


Fig. 2. Extracts from atherosclerotic plaques preserved in RNAlater are suitable for protein analysis by ELISA-based assays.

(A), Correlation matrix, with each color box in the matrix representing the interanalyte *R* value between a pair of protein analytes (from log-transformed values). The proteins are listed in the same order right-to-left as top-to-bottom. (B), Extracts from 4 plaques preserved by the flash-freezing method, identified with the symbols □, ◆, Δ, or ▼, were assayed for RANTES, TNFRII, and MPO content, either with no addition, or spiked with a range of RNAlater concentration. The downward-pointing arrows indicate extracts assayed at a concentration of RNAlater equivalent to the mean concentration of RNAlater determined in extracts of 85 independent plaque samples collected into RNAlater.

trogen and transferred into a TT1 homogenization tube for RNA extraction.

Grinding-extraction method for tissue splitting (GEMS) method. A ceramic pestle and mortar (CoorsTek) were cleaned with soap and warm water, rinsed with distilled water, treated with RNaseZap® wipes (Ambion), rinsed with nuclease free ultrapure water (Gibco) and then with 70% ethanol in RNase-free water, dried in a tissue culture hood under ultraviolet light, covered with RNaseZap-treated aluminum foil, and stored at room temperature. Plastic weighing dishes, prepared similarly, were used to manipulate plaque powder before protein isolation.

The GEMS method is illustrated in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue11. The pestle and mortar were prechilled on dry ice, and flash-frozen tissue was transferred to the mortar. Tissue collected in RNAlater was removed from liquid, weighed, and then transferred to the mortar. Approximately 5 mL of liquid nitrogen was applied twice. Samples were then ground to a uniform fine powder. Every 2–3 min more liquid nitrogen was added. A disposable, sterile, DNase- and RNase-free spatula (Corning) was used to collect powder and divide it into equal portions. For archiving, powder was transferred to a prepared and prechilled (dry ice) 2-mL cryovial and weighed.

TMAD method. We designed a custom, disposable milling tube tolerant of impact at subzero temperatures and with an optimal internal geometry. The tube was manufactured of Teflon and injection molded at 300 °C, with a polished interior and silicon seal (see online Supplemental Fig. 2A and Fig. 3A). Tubes were not pretreated before use. A frozen plaque sample and a dry, chilled stainless steel bead were placed inside the milling tube (see online Supplemental Fig. 3A), which was placed in a prechilled adaptor block (see online

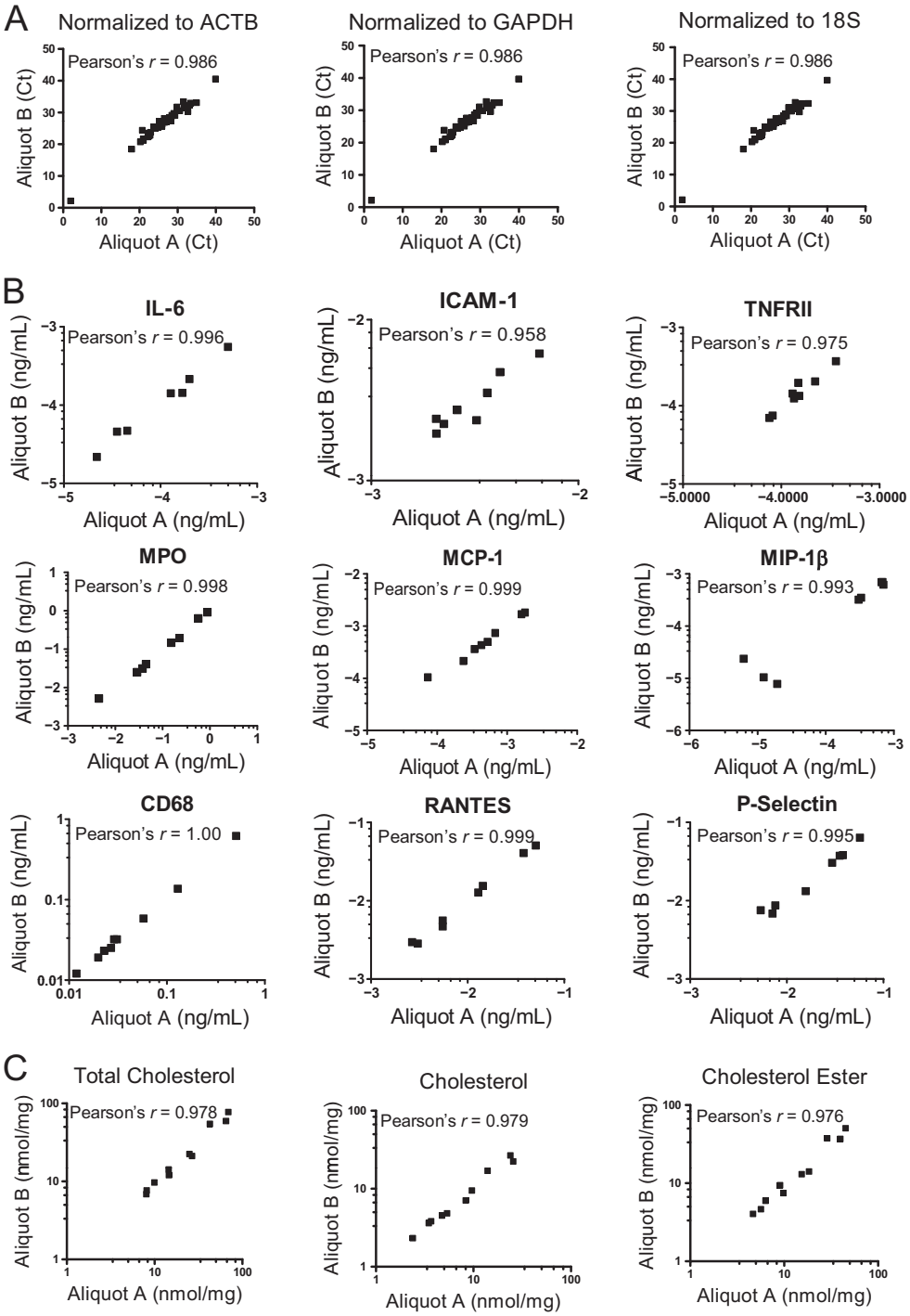


Fig. 3. Evaluation of compositional homogeneity of aliquots generated by the tissue-splitting method.

(A), Each data point represents the cycle threshold (Ct) values obtained from quantitative reverse-transcription PCR expression analysis of 1 of 45 genes from aliquots A and B from a single plaque sample, independently normalized to 1 of 3 housekeeping genes. (B), Each data point represents the concentrations of the indicated protein in aliquots A and B from 1 of 8 plaques analyzed. (C), Each data point represents the concentrations of the indicated lipid in aliquots A and B from 1 of 10 plaques analyzed.

Supplemental Figs. 2B and 3B) designed to fit the Qiagen TissueLyser (see online Supplemental Fig. 3C). The milling-tube/adaptor-block assembly was dipped in a liquid nitrogen bath and then loaded onto the TissueLyser and milled at 30 kHz in 30-s intervals 3–5 times, depending on sample size and consistency. Samples were periodically immersed in liquid nitrogen. When tissue had been milled to a fine, homogeneous powder, it was split by use of a custom-made apparatus consisting of a disposable surface, the splitter tray, and a receiving block, nested in dry ice (see online Supplemental Fig. 2, C and D, and Fig. 3, D–F). Powder division was accomplished by pushing it through the splitting tray's holes into receiving cryovials below. Samples were stored at −80 °C. We tracked all tube-to-tube transfers, as well as tissue powder masses for each split, using a Microsoft Access database.

RNA EXTRACTION AND ANALYSIS

After standard method or TMAD tissue processing. We mixed 1 mL of TRIzol® reagent (Invitrogen) per 100 mg of tissue material with a routine tissue-powder weight of 50–100 mg, and homogenized the mixture using a Fisher Scientific PowerGen 1000 with disposable Omni hard tissue generators (Omni International). Total RNA was isolated from the aqueous phase with Wizard SV96 plates (Promega) according to the manufacturer's instructions.

After GEMS tissue processing. We added 1 mL of TRIzol reagent to 50–100 mg of tissue powder in the mortar. The mixture was stirred, covered with RNaseZap-cleaned aluminum foil, put at room temperature to thaw, and then transferred into a 2-mL DNase/RNase-free tube (Eppendorf Biopur). The sample was homogenized by using a hand-held Biospec Tissue Tearor (power setting 15, 7-mm probe) and moving the probe up and down in the tube for 15 s (1 cycle). Depending on sample size, 3–5 cycles of homogenization were required, with 1-min incubation on ice between cycles. RNA was isolated by using the RNeasy Mini Kit (Qiagen). Briefly, we added 0.2 mL of chloroform to the homogenate, which was then vortex-mixed and centrifuged (15 000*g* for 10 min). We then added 200 μL from the aqueous phase to 700 μL of Qiagen RLT buffer in a new tube and processed this mixture according to manufacturer's instructions.

RNA evaluation. The sample QC system used a fluorescence-based dye, RiboGreen (Invitrogen), to quantify RNA, according to manufacturer's instructions. An automated microtiter plate–based QC system with robotic loading of samples onto Agilent Bioanalyzer Pico chips was used. All liquid transfers were performed on a Beckman Biomek FX dual-pod instrument with a Span 8 and 96-channel P200 head. In some cases RNA concentration was determined by NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Scientific) according to manufacturer's instructions. Analysis of RNA integrity was performed on an Agilent 2100 Bioanalyzer microfluidics-based platform (Agilent) with RNA Pico chips according to manufacturer's instructions. The 28S/18S ribosomal RNA ratio and RNA integrity number (RIN) scores were acquired from the Agilent Technologies 2100 Expert software package.

Quantitative PCR. TaqMan low-density array (TLDA) (Applied Biosystems) cards were custom configured into 8 identical 48-gene sets. Genes were selected on the basis of published studies of atherosclerotic plaque composition. Each cDNA sample (100 μL) was added to an equal volume of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems). The mixture was transferred into a loading port on a TLDA card, which was centrifuged and sealed. Amplification was performed by using an Applied Biosystems Prism 7900HT sequence-detection system. Expression values were calculated by using the comparative Ct method as described (User Bulletin No. 2, Applied Biosystems). The TLDA genes were: phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (*PLA2G7*); dual specificity phosphatase 1 (*DUSP1*); chemokine (C-C motif) ligand 2 (*CCL2*); arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*); adenosylhomocysteinase (*AHCY*); prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (*PTGS2*); heme oxygenase (decycling) 1 (*HMOX1*); myeloperoxidase (*MPO*); chemokine (C-C motif) receptor 1 (*CCR1*); chemokine (C-C motif) ligand 7(*CCL7*); *4342379–18S*; vascular cell adhesion molecule 1 (*VCAM1*); CD5 molecule-like (*CD5L*); FBJ murine osteosarcoma viral oncogene homolog (*FOS*); ATP-binding cassette, sub-family A (ABC1), member 1 (*ABCA1*); tumor necrosis factor (*TNF*); hydroxycarboxylic acid receptor 3 (*HCAR3*); platelet-activating factor receptor (*PTAFR*); chemokine (C-C motif) ligand 3 (*CCL3*); matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) (*MMP9*); matrix metallopeptidase 1 (interstitial collagenase) (*MMP1*); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); chemokine (C-X-C motif) ligand 2 (*CXCL2*); interleukin 8 (*IL8*); arachidonate 5-lipoxygenase (*ALOX5*); cystathionine-beta-synthase (*CBS*); cysteinyl leukotriene receptor 2 (*CYSLTR2*); CD163 molecule (*CD163*); early growth response 2 (*EGR2*); chemokine (C-C motif) ligand 5 (*CCL5*); leukotriene B4 receptor (*LTB4R*); ADAM metallopeptidase with thrombospondin type 1 motif, 1 (*ADAMTS1*); cathepsin L1 (*CTSL1*); actin, beta

(*ACTB*); interleukin 6 (interferon, beta 2) (*IL6*); C-reactive protein, pentraxin-related (*CRP*); nuclear receptor subfamily 4, group A, member 1 (*NR4A1*); interleukin 1, alpha (*IL1A*); peptidylprolyl isomerase A (cyclophilin A) (*PPIA*); proliferating cell nuclear antigen (*PCNA*); secreted phosphoprotein 1 (*SPP1*); selectin E (*SELE*); scavenger receptor class B, member 1 (*SCARB1*); cysteinyl leukotriene receptor 1 (*CYSLTR1*); phospholipase A2, group X (*PLA2G10*); nitric oxide synthase 2, inducible (*NOS2*); integrin, alpha X (complement component 3 receptor 4 subunit) (*ITGAX*); and intercellular adhesion molecule 1 (*ICAM1*).

PROTEIN EXTRACTION AND ANALYSIS

Protein isolation after GEMS or TMAD. Tissue powder (50–100 mg) was mixed with 300 μL of ice-cold PBS/1% CHAPS, transferred to a 10-mL glass tube (Fisher) and combined with 300 μL of PBS/CHAPS rinsate from the original powder container. Samples were homogenized in a Covaris E200. After 1 round of homogenization, the sample was centrifuged at 2143*g* for 30 min, the supernatant was collected, additional buffer was added to the pellet, and a second round of homogenization and centrifugation was carried out. The supernatants were combined and frozen in aliquots at −80 °C.

Protein analysis platform 1. Interleukin (IL)-1β, macrophage inflammatory protein-1β (MIP-1β), IL-6, monocyte chemotactic protein-1 (MCP-1), regulated on activation normal T-cell expressed and secreted (RANTES), C-reactive protein (CRP), CD68, tumor necrosis factor receptor-II (TNFRII), intercellular adhesion molecule-1 (ICAM-1), and myeloperoxidase (MPO) were quantified by immunoassays on a Meso Scale Sector Imager 6000 (Meso Scale Discovery).

Protein analysis platform 2. Protein extracts were sent to Rules-Based Medicine (Austin, TX) for analysis with the human MAP (multianalyte profiling) panel version 1.6 (http://www.rulesbasedmedicine.com).

Determination of sulfate content. We determined sulfate concentration in extracts prepared from plaque specimens preserved in RNAlater by using a QuantiChrom™ sulfate assay kit according to manufacturer's instructions.

LIPID EXTRACTION AND ANALYSIS

Pellets from protein extraction were extracted with chloroform:methanol (2:1) according to the method of Folch et al. *(3)* as modified by Zhu et al. *(4)*. Sample size was determined by size of the sample used for protein extraction.

PROCESSING LARGE NUMBERS OF PERIPHERAL PLAQUE SAMPLES

Samples ≤20 mg were used only for protein extraction; samples between 40 and 100 mg were subjected to TMAD and split for RNA and protein extraction; samples ≥100 mg were subjected to TMAD and split 3 ways, for archiving and RNA and protein extraction.

COMPARISON OF LIPID CONTENT IN LEFT AND RIGHT LOWER EXTREMITIES

Patients in the placebo arm of the study (Clinicaltrials.gov: NCT00720577) were randomized to a sequence, and plaque was collected from either the right leg first and then the left leg or the left leg first and then the right leg. Six weeks elapsed between collections.

STATISTICAL ANALYSES

Fixed bias, proportional bias, and the imprecision between GEMS and TMAD were compared by using ANOVA. For each split plaque sample, a linear measurement error model was used *(5)*.

The Mann–Whitney test was used to assess the significance of differences between the 2 processing methods when RNA quality and yield from TMAD were compared with those obtained with the standard method.

A 1-way ANOVA (with the term left or right leg) was used to assess differences in lipid content in the left vs right leg by using the proc mixed command in the SAS (Statistical Analysis System) application. The subject term was assumed to be random.

Results

OPTIMIZATION OF TISSUE COLLECTION AND PRESERVATION PROTOCOL

To identify a collection protocol yielding high-quality RNA, we compared 2 methods: flash freezing in liquid nitrogen or immersion in RNAlater. In both cases the tissue was processed and RNA extracted by the standard method. RNA obtained from flash-frozen samples exhibited an 18% pass rate (passing criteria: RIN >5, 28/18S rRNA ratio 0.75–3.02) vs a 90% pass rate for tissue preserved in RNAlater.

Next we evaluated the compatibility of tissue preservation in RNAlater with protein extraction by performing comparative interanalyte correlation analysis using data from quantification of 10 proteins in samples preserved either by flash freezing (n = 61, processed by standard method) or in RNAlater (n = 30, processed by TMAD) (Fig. 2A). The results showed similar patterns of correlations of proteins present when samples were prepared by either method.

RNAlater contains a high concentration of ammonium sulfate *(6)*. We measured sulfate content in 85 plaque extracts and determined that the RNAlater con-

centration did not exceed 0.4% at dilutions required for measuring IL-1β, IL-6, MCP-1, and RANTES, and 0.04% at dilutions required for measuring CRP, CD68, ICAM-1, MPO, TNFRII, and MIP-1β. To establish that RNAlater was compatible with quantitative analysis of these proteins, we prepared extracts from 4 different plaques preserved by flash freezing and then measured the concentrations of RANTES, TNFRII, and MPO in the absence of RNAlater and after adding increasing amounts of RNAlater to the assay (Fig. 2B). Concentrations of RNAlater up to 0.5% in extracts had no discernable effect on apparent protein concentration in these extracts. The results for assays of IL-1β, IL-6, MCP-1, CRP, CD68, ICAM-1, and MIP-1β were essentially identical. We therefore used RNAlater to preserve samples.

MANUAL GRINDING DOES NOT COMPROMISE RNA QUALITY

We evaluated a method of grinding frozen plaque samples into fine powder with a mortar and pestle (GEMS). The GEMS method is technically simple and takes place in a buffer-free environment (see online Supplemental Fig. 1). RNA quality was essentially the same after tissue processing by either the standard method or by GEMS (mean 28S:18S, rRNA ratio 1.7). Analysis of 39 plaque samples from superficial femoral arteries and 7 samples from popliteal arteries confirmed that RNA of consistently high quality can be obtained by GEMS: the mean (SD) values of the 28S:18S rRNA ratio and RIN for the superficial femoral arteries were 1.5 (0.24) (n = 39) and 7.4 (2.1) (n = 36), respectively, and for popliteal arteries were 1.4 (0.19) (n = 7) and 7.6 (0.59) (n = 5).

SAMPLE ALIQUOTS GENERATED BY GEMS ARE COMPOSITIONALLY EQUIVALENT

To demonstrate that GEMS yielded aliquots of equivalent composition compatible with the analysis of biomarkers of different natures, we sequentially evaluated RNA composition in 2 aliquots from 4 plaques, protein composition in 2 aliquots from 5 plaques, and lipid composition in 2 aliquots from 10 plaques. For each evaluation a different set of plaques was used (see online Supplemental Fig. 1G).

Total RNA extracted from 2 aliquots (A and B) of 4 plaque samples was analyzed for expression of 45 genes by quantitative reverse-transcription PCR. The mean correlation coefficient between the A and B aliquots for each plaque was 0.987 (range 0.982–0.995). A representative analysis of 1 plaque sample is shown in Fig. 3A.

In different samples we examined the protein composition of aliquot pairs. The mean correlation coefficient between the A and B aliquots for the 5 plaque samples examined was 0.997 (range 0.988–1.000) for a panel of proteins in protein analysis platform 1 and 0.978 (range 0.955–0.998) for a panel in platform 2.

To ensure that the splitting technique was suited for analysis of different types of proteins, we performed correlation analysis for 9 proteins representing different categories (secreted: RANTES, IL-6, MCP-1, MIP-1β; membrane: CD68, TNFRII, P-selectin, ICAM1; intracellular: MPO) measured in 2 aliquots from 8 samples (Fig. 3B). The correlations between aliquots for all proteins showed that the splitting technique was suited for analysis of different types of proteins. Observed differences in composition between sample aliquots for individual proteins were within the variability of technical performance (characterized by CV values) of their corresponding assays (ranging from 23% for IL-1β to 9% for CD68). We observed similar correlations when we compared the lipid content in 2 aliquots from another set of 10 plaque samples (Fig. 3C).

To test if the overall composition of sample aliquots originating from the same parent sample were similar, we performed hierarchical clustering *(7)* of protein abundance data for 89 proteins measured in 2 aliquots from 5 different plaque specimens processed by GEMS. The data derived from aliquot pairs of the same parent specimen clustered together and were compositionally almost identical, with a mean similarity of 99.5% (Fig. 4).

DEVELOPMENT OF A SEMIAUTOMATED METHOD FOR TISSUE SPLITTING

To improve tissue-processing efficiency and standardization, we developed a semiautomated TMAD method. TMAD featured a sealed milling vessel with a bead that delivers a rapid, electronically controlled blunt impact force on frozen tissue (see online Supplemental Figs. 2 and 3). All tissue-handling steps occurred at subzero temperatures to ensure sample integrity and keep tissue brittle.

To split fine tissue powder into multiple aliquots, a custom system was designed for use at subzero degrees centigrade temperature in a biosafety cabinet. The system was designed to accommodate the inversion of the milling tube onto the chilled splitter tray surface for the recovery of the milled tissue powder (mean rate 98%) and its division into aliquots (see online Supplemental Figs. 2, C and D, and Fig. 3, E and F). This design allowed efficient processing and splitting of tissue, with mass ranging from approximately 25 to 250 mg in 1 tube.

COMPARISON OF PERFORMANCE CHARACTERISTICS BETWEEN GEMS AND TMAD

We used ANOVA to compare fixed bias, proportional bias, and imprecision between GEMS and TMAD.

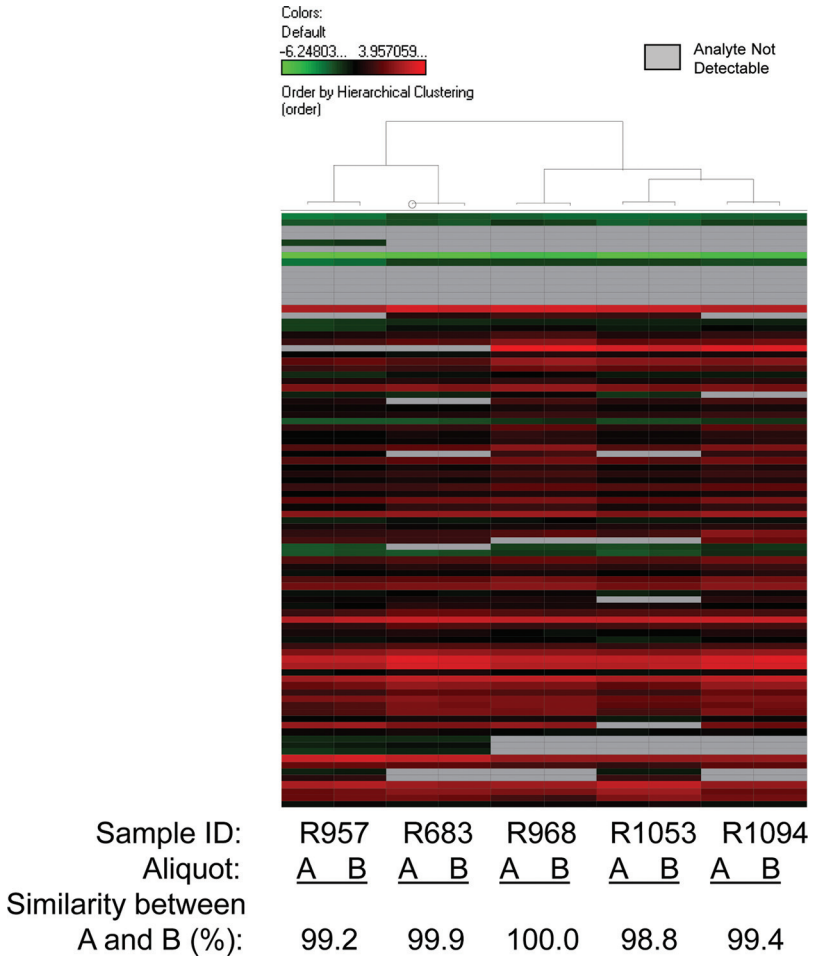


Fig. 4. Molecular analysis results demonstrated that the composition is nearly identical for aliquots created from a single specimen by tissue splitting by GEMS.

Data from measurement of 89 protein analytes in aliquots A and B from 5 samples were used for analysis by unsupervised clustering.

Four to 5 plaque samples were split by GEMS or TMAD and concentrations of protein biomarkers were determined in each sample aliquot by using protein analysis platforms 1 or 2, respectively (Fig. 5, A and B). For each split plaque specimen a linear measurement error model was used *(5)*. There was no statistically significant difference between GEMS and TMAD in the bias and imprecision for a panel of analytes measured on platform 1 (the fixed bias, proportional bias, and CV for GEMS were 0.030%, 0.004%, and 6.0%, respectively, and for TMAD were 0.019%, −0.01%, and 7.6%), but there was a significant difference in the imprecision for a panel of analytes measured on platform 2 (GEMS: 0.037%, 0.002%, 12.0%; TMAD: −0.052%, 0.005%, 25.9%). However, the number of differences between sample splits falling outside 2-fold difference limits was small (<5%) for all analytes measured (Fig. 5, A and B). Thus, GEMS and TMAD exhibited comparable performance characteristics.

LARGE-SCALE APPLICATION OF THE TISSUE-SPLITTING METHOD

To establish the large-scale clinical applicability of TMAD, milled powders from 293 peripheral plaques from a phase 0 clinical study (Clinicaltrials.gov: NCT00720577) were generated and divided into aliquots for analysis of mRNA. We observed a small but significant ($P = 0.003$) increase in a median RIN number (7.9 vs 7.2) for specimens processed by TMAD (n = 189) compared with specimens collected during methods-development studies and processed by the standard method (n = 30) (Fig. 5C). There was also a very significant, almost 2-fold increase in the median

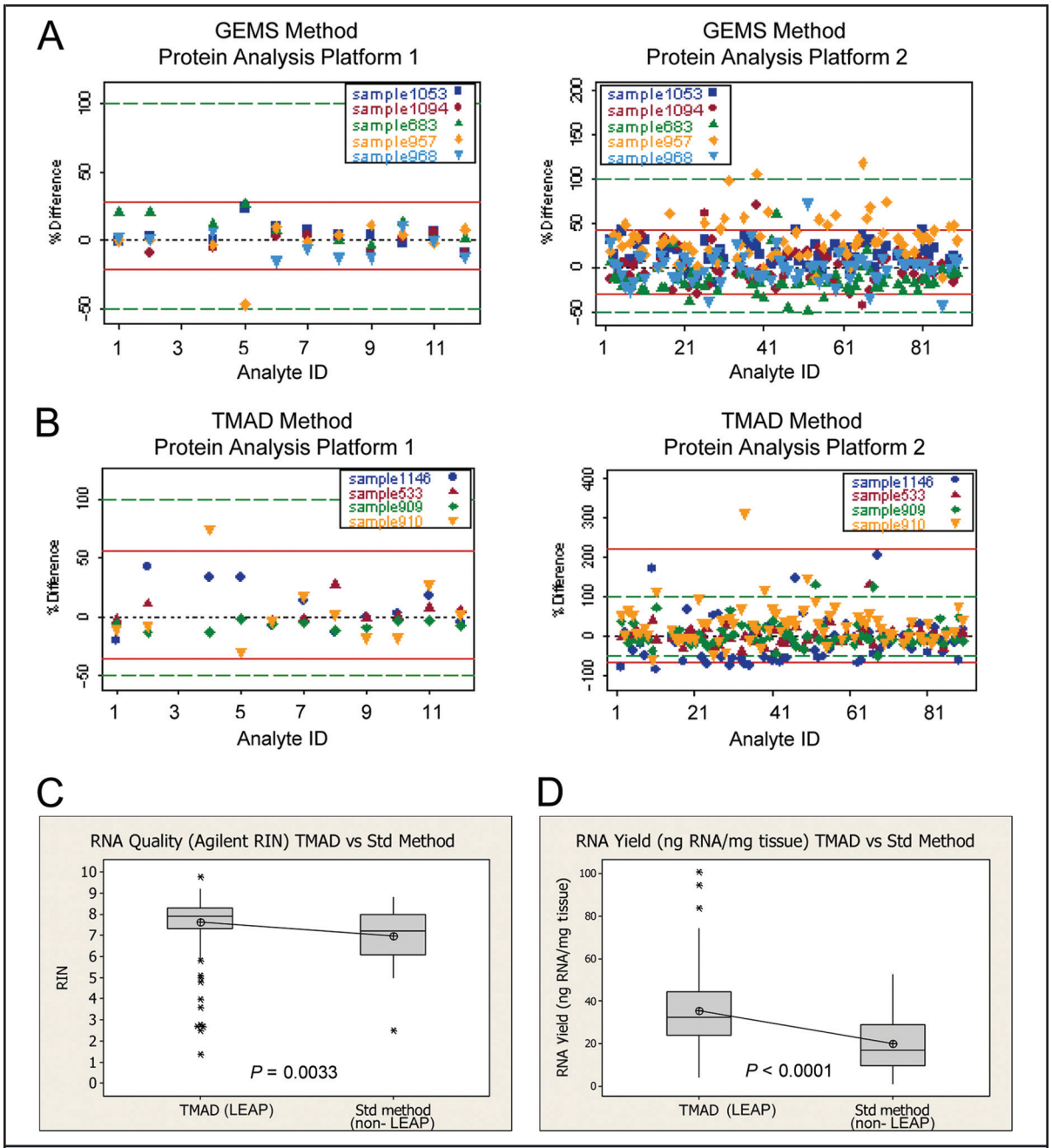
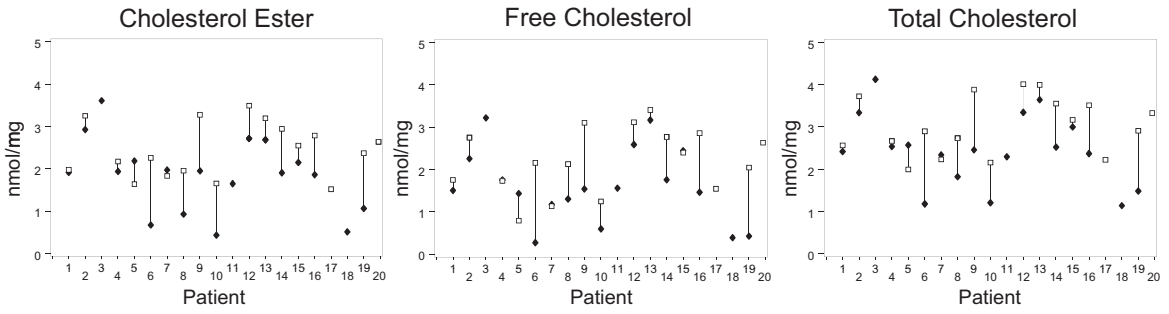


Fig. 5. Comparison of GEMS, TMAD, and standard (Std) method performance characteristics.

(A), GEMS; (B), TMAD. The % difference between the amounts of each protein found in each pair of aliquots is shown as a colored symbol. The solid red lines represent data-driven agreement limits, the dashed green lines represent 2-fold difference limits, and the dotted line indicates a perfect agreement between any 2 aliquots of same sample. RNA quality (C) and yield (D) were compared between the TMAD and standard methods of tissue preparation. In both (C) and (D) the middle horizontal line represents the mean, the upper and lower horizontal lines represent the upper and lower 95% CIs, the vertical lines represent the SDs from each sample population, and the asterisks represent samples falling greater than 2 SDs away from the mean. LEAP, Lower-Extremity Assessment Project.



Parameter	Mean right leg	Mean left leg	Mean difference	*P* value
Cholesterol ester (nmol/mg)	1.83383	2.43935	0.6479	0.001

Parameter	Mean right leg	Mean left leg	Mean difference	*P* value
Free cholesterol (nmol/mg)	1.60156	2.21024	0.6317	0.004

Parameter	Mean right leg	Mean left leg	Mean difference	*P* value
Total cholesterol (nmol/mg)	2.43444	3.03435	0.6315	0.002

Fig. 6. Comparison of cholesterol and cholesterol ester composition in peripheral plaque samples excised from right and left lower extremities of individual patients.

Right-leg (♦) and left-leg (□) lipid quantities for each patient are connected by a line. Five patients had samples from only 1 leg.

yield of RNA from specimens processed by TMAD (n = 194) compared with those processed by the standard method (n = 38) (Fig. 5D). All samples compared were obtained from RNAlater preserved tissue.

We used these samples to assess aspects of biological variability of plaque composition by comparing the lipid content of lesions excised from the left and right lower extremities. We observed a significantly higher mean content of cholesterol ester, free cholesterol, and total cholesterol (32%, 40%, and 25% increase, respectively) in atherosclerotic lesions excised from left lower extremities (Fig. 6).

Discussion

We designed, engineered, and validated a semiautomated technique (TMAD) for splitting tissue into compositionally identical aliquots. This technique enabled cost-effective analysis of biomarkers requiring noncompatible tissue extraction protocols in single samples. TMAD standardizes the milling and splitting processes and allows increased throughput and a high rate of sample recovery from small tissue specimens (98%). All materials that contact tissue are disposable, eliminating the risk of cross contamination. TMAD is compatible with quantitative analysis of mRNA, protein, and lipid content, enabling detailed characterization of molecular composition of tissue without the need to collect separate specimens for each analysis.

Preserving specimen quality is the prime goal of optimizing tissue collection conditions *(8)*. We observed a dramatic improvement in the quality of RNA when we preserved plaque in RNAlater. The simplicity of this collection method makes it well suited for applications requiring a balance between implementation in a clinical setting and analysis in a research laboratory. In addition, our data demonstrated that tissue preservation in RNAlater is compatible with quantification of protein and lipid analytes in tissue extracts. This result confirms and extends previously reported findings indicating that RNAlater preservation is suitable for ELISA-based methods *(9)*.

Few protocols for simultaneous isolation of RNA, DNA, and proteins have been described, and none were semiautomated with high throughput *(10, 11)*. These published methods relied on strong denaturing agents such as guanidine isothiocyanate without or with phenol to prevent RNA degradation during extraction. This approach would be incompatible with assays requiring native protein conformation. Commercially available methods such as NucleoSpin® RNA/Protein (Macherey-Nagel) or AllPrep (Qiagen) have similar limitations. By creating compositionally identical aliquots from a parent specimen, TMAD enables selection of optimal conditions for extraction and analysis of biomolecules of interest.

The GEMS and TMAD techniques allowed the grinding (GEMS) or milling (TMAD) and dividing of plaque specimens into homogenous aliquots of fine powder at subzero temperatures. Temperature control not only mitigates the risk of analyte degradation during processing but also keeps the tissue brittle, facilitating grinding and milling. We observed a small but significant increase in median RIN number as well as a very significant almost 2-fold increase in the median yield of RNA for specimens preserved in RNAlater and

processed by TMAD compared with the standard method of tissue processing (Fig. 5, C and D). It is possible that TMAD's much lower operating temperatures (−70 to −180 °C) help stabilize RNA during processing, and that creating a larger surface area by milling the tissue into powder enhances extraction efficiency. Additional advantages of TMAD over GEMS include greater recovery of material by TMAD, reduced time cleaning the apparatus, and less manual effort and reduced potential for repetitive motion injuries. Because TMAD is designed to work in a closed system (microtube) and is easier to perform in a safety cabinet, it reduces a risk of sample or operator contamination.

Ensuring equivalency of composition of aliquots generated from the parent specimen was a critical component of GEMS/TMAD development. The mean correlation coefficient between the compositions of aliquots generated by GEMS was near 1 for all analytes tested. The residual variability between aliquots for individual proteins measured did not exceed variability of analytic platforms used for their measurement.

Some patients with peripheral artery disease (PAD) develop atherosclerotic plaque in both lower extremities (bilateral PAD). To explore the epidemiology of bilateral PAD progression we used samples processed by TMAD to analyze lipid composition in plaques from left and right legs in patients undergoing plaque excision with a SilverHawk catheter. We observed a significantly higher content of cholesterol ester, free cholesterol, and total cholesterol in lesions from left extremities (Fig. 6). This result suggests that progression of bilateral PAD is not symmetrical and that plaque has a tendency to develop in left extremities earlier and/or more rapidly. Variability and differences in composition of left and right lesions has important practical implication that should be taken into consideration when clinical trials are designed to be performed in a population of patients with bilateral PAD.

In conclusion, we have developed a technique for splitting tissue into multiple compositionally identical aliquots. This technique can simplify collection of clinical samples and their interrogation by use of various analytical techniques, and contribute to deeper understanding of biological processes in human diseases.

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