REPLY TO MÄKINEN AND ALA-KORPELA:

Small-scale but accurate metabolomics with high reproducibility for identifying age-related blood metabolites

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Human aging is a highly complex biological process exhibiting great individual variation. Although distinguishing young and elderly people visually is easy and intuitive, it is still not possible to distinguish human blood samples from young and elderly donors. Nonetheless, chemical biology holds great promise for identifying age-related metabolites, quantitatively and qualitatively.

As the letter by Mäkinen and Ala-Korpela (1) points out, various publications have strongly recommended analysis of more than 1,000 samples to draw reliable conclusions about identification of biomarkers; however, increasing the number of samples does not necessarily increase data reproducibility and reliability. Experimental design can be more important than sample size, depending upon the nature of the phenomena being investigated. For example, analysis of a small sample ($n = 42$) recently led to the discovery of a prostate tumor biomarker (2).

Our incentive for the present (3) and previous (4) studies was to develop standard experimental procedures to identify and precisely quantify human age-related blood metabolites with the use of a limited number of samples. To this end, our intent was to increase the reproducibility and accuracy of measuring blood metabolites, which are highly unstable. Rapid quenching of samples, whole-blood analysis without centrifugation, and use of a hydrophilic interaction liquid chromatography column (3, 4) were essential to the success of this effort. Blood donor volunteers of various ethnic backgrounds came to the university hospital laboratory to facilitate immediate sample preparation.

Additionally, our deliberate exclusion of middle-aged donors (40–70 y old) from the study gave us a clearer age difference between young (average = 29 y) and elderly (average = 81 y) donors. The combination of comprehensive coefficient of variation (CV) analysis and red blood cell (RBC) metabolomics enabled us to identify 51 previously unreported metabolite CVs. Ultimately, we presented evidence that a small number of blood samples ($n = 30$) clearly indicated 14 possible age-related metabolites (P values from 0.0004 for *N*-acetyl-arginine to 0.046 for NAD^+), with rigorous precautions to prevent variation in sampling and measurements (3). Six of these 14 candidates are RBC-enriched, suggesting that RBC metabolomics is highly valuable.

Our findings are consistent with previous studies (5, 6) that also used relatively small sample sizes, suggesting the reproducibility of our results. Recent reports also support the notion that NAD, leucine, and isoleucine are involved in biological aging (7, 8). Furthermore, Pearson's correlation coefficients demonstrated significant correlations among several age-related compounds, including citrulline, *N*-acetyl-arginine, dimethyl-guanosine (implicated in renal function), NAD, NADP (redox-related), leucine, and isoleucine (muscle maintenance). Their correlation strongly supports the physiological significance of our findings and the importance of using correlation functions as well as CVs (high values of correlation: 0.83 between leucine and isoleucine, 0.84 between citrulline and *N*-acetyl-lysine, 0.73 between carnosine and $NADP^+$, etc.).

We agree with Mäkinen and Ala-Korpela about the value of longitudinal studies (1), and about the utility of large sample sizes for some types of study. However, although on the basis of sample size alone, they have summarily dismissed our results as attributable to chance, they have failed to explain how such high correlation coefficients could be expected among compounds identified by chance, if the experimental methods and sample sizes were inappropriate, and they have also ignored the wealth of supporting information presented in our paper. We think that such "doctrinal" pronouncements do not serve the field well and that more thoughtful analysis is required.

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The authors declare no conflict of interest.

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