Reproducibility of serum inflammatory biomarkers in an epidemiological study - Singapore Chinese Health Study (SCHS)

Guillaume Onyeaghala[1], Helene Barcelo[2,3], Heather H Nelson[1,2], Bharat Thyagarajan[2,3], Yian-Min Yuan[4], Anna Prizment[1,2]

[1] University of Minnesota Div. of Epidemiology & Community Health, School of Public Health, [2] Masonic Cancer Center,
[3] Department of Medicine, School of Medicine, University of Minnesota; [4] Div. of Cancer Control and Population Sciences, University of Pittsburgh, Pittsburgh, PA

Introduction

- There is a growing need for improved screening and diagnostic methods based on blood-based biomarkers to better elucidate hypotheses in epidemiology.
- Low-grade systemic inflammation has been shown to be associated with obesity, and also predisposes people to the development of future colorectal cancer, but previous studies focused on a small number of inflammatory biomarkers.
- Several studies have demonstrated that higher power of discrimination can be obtained by combining more than one biomarker, However immunoassays used to quantify multiple proteins (such as bead-based and planar arrays) require extensive optimizations to eliminate antibody cross-reactivity.
- The proximity Extension Assay (PEA) is based on pairs of antibodies that are linked to oligonucleotides having slight affinity to one another (PEA probes). Upon target binding the probes are brought in proximity, and the two oligonucleotides are extended by a DNA polymerase forming a new sequence that now acts as a unique surrogate marker for the specific antigen.
- By virtue of the proximity requirement for template formation and the stringency attained from the qPCR readout, antibody cross-reactivity is unlikely to be detected and cause problems in PEA.

Goal of the study

- Demonstrate the reproducibility of O-link inflammatory panel, using the Proseek Multiplex Inflammation kit in 30 serum samples collected from initially cancer-free participants of the Singapore Chinese Health Study (SCHS)

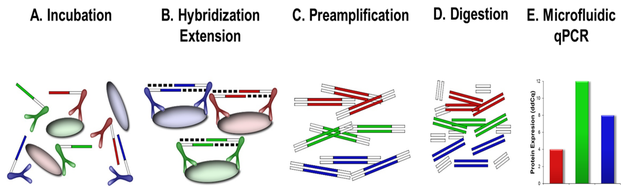

Figure 1. Design and description of 96-plex PEA (Taken from Assarsson *et al. 2014*) (A) 94 pairs of specific antibodies are equipped with oligonucletotides (PEA probes) and mixed with an antigen-containinig sample. (B) Upon sample incubation, all proximity probe pairs bind their specific antigens, which brings the probe oligonucleotides in close proximity to hybridize. The oligonucleotides have unique annealing sites that allows pair-wise binding of matching probes. Addition of a DNA polymerase leads to an extension and joining of the two oligonucleotides and formation of a PCR template. (C) Universal primers are utilized to preamplify all 96 different DNA templates in paralell. (D) Uracil-DNA glycosylase partly digests the DNA templates and remove all unbound primers. (E) Finally each individual DNA sequence is detected and quantified using specific primers in by microfluidic qPCR.

Methods

Study Design:

- The SCHS is a population-based cohort of 63,257 Chinese women and men aged 45-74 years in Singapore.
- At recruitment (1993-98), data on demographic characteristics and lifestyle factors (including weight and height) were obtained through in-person interviews
- Serum, buccal and urine samples were collected from 32,546 participants, frozen, and stored at -80° C.
- Incident cancer cases were identified through the population-based Singapore Cancer Registry through 2010.
- Cases and controls were matched individually by age, sex and dialect (Hokkien vs Cantonese).

Measurements :

- We randomly selected 15 samples from participants who subsequently developed CRC and 15 samples who did not develop cancer until the end of follow-up
- The levels of biomarkers were measured using the Proseek Multiplex Inflammation kit in the University of Minnesota Genomics Center (UMGC) and Dr. Thyagarajan's lab.

Statistical analysis:

- All analyses were conducted using the GenEx 6 Software for qPCR data processing and analysis (MultiD Analyses AB, Sweden) and SAS v9.3
- The measurements of inflammatory markers in Dr. Thyagarajan's lab and UMGC lab were compared using the following procedures:
  - Bland-Altman plots to assess the magnitude of disagreement, check for bias and spot outliers.
  - The intra-class correlation coefficients (ICCs) were calculated using the analysis of variance (ANOVA) procedureby dividing the between-subject variance by the sum of the between-subject variance and between-lab variances.

Results

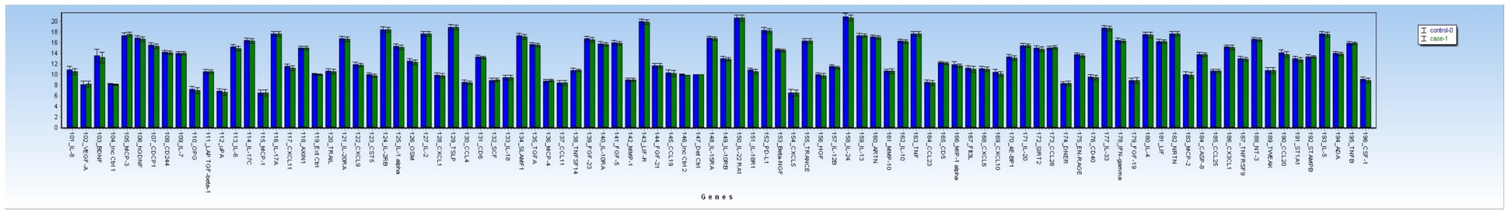

Figure 2. Average NPX values and 95% CI comparing cases and control for each inflammatory biomarker

- X-axis – each column corresponds to an inflammatory biomarker (overall – 92 biomarkers)
- Y-axis: Normalized Protein eXpression (NPX), is an arbitrary unit in Log2 scale
- Data pre-processing (normalization) is performed to minimize both intra- and inter-assay variation
- The NPX scale is inversed to that of Cq: a high NPX value represents a high protein concentration, and an increase of 1 NPX corresponds to a 2-fold increase in protein amount.

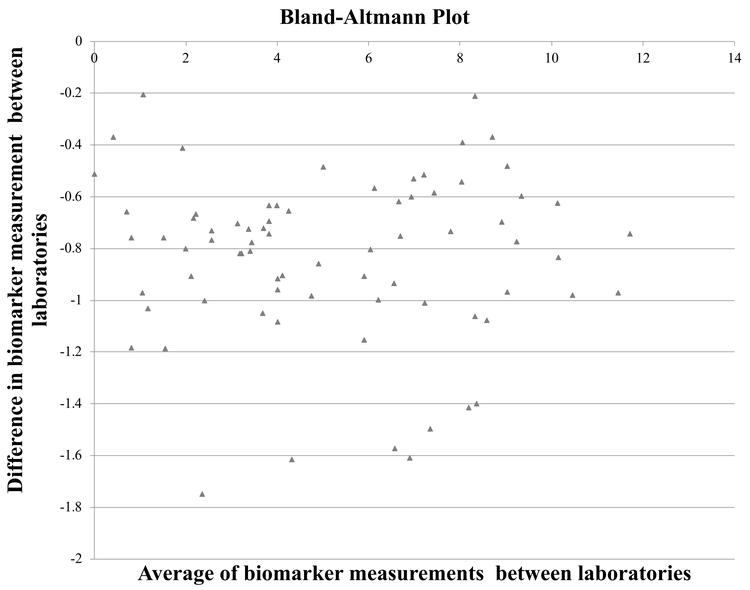

Figure 3. Bland-Altmann Plot comparing the average of biomarker measurements between the MDL and UMGC laboratories versus the differences in biomarker measurements across 30 samples.

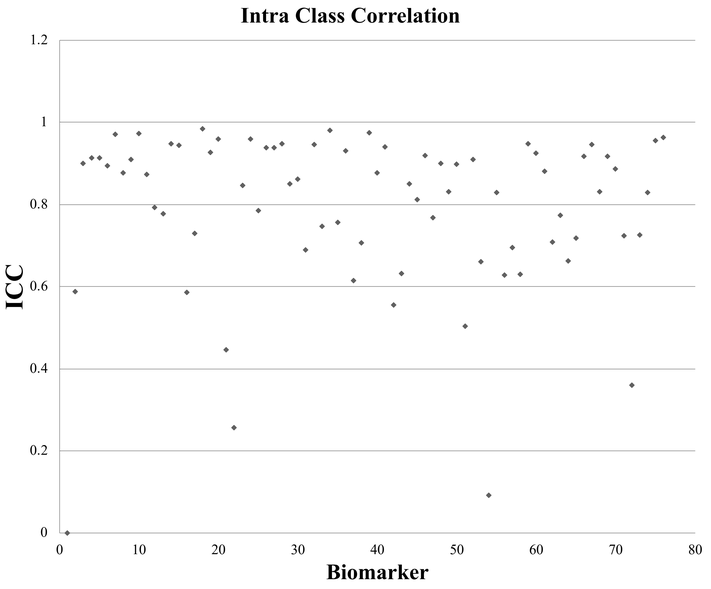

Figure 4. Intra Class Correlation plot comparing biomarker measurements between the MDL and UMGC laboratories across 30 samples.

Results

- There was evidence of systematic difference (fixed bias) between the UMGC and MDL laboratories, with NPX values consistently higher in the UMGC run.
  - This bias may be attributable to differences in pipetting methods between laboratories, since the assay only requires 15 uL of sample
- 90% of all compared biomarkers had an intraclass correlation coefficient greater than 0.5. Furthermore, 74% of the compared biomarkers had a intraclass correlation coefficient between 0.7 and 1
- The values of all biomarkers were similar for cases and controls.

Conclusions

- The proximity extension assays offer reproducible measurements between laboratories
- Large scale studies using multiple assays are needed for further evaluations

Future directions

- Create Inflammatory Scores using principal component analysis.
- Study the association of individual biomarkers and Inflammatory Scores with colorectal cancer risk factors: age, sex, dialect and BMI

Acknowledgements

This study was supported by the UMGC/Olink Proseek Multiplex Pilot Grant Program. Anna Prizment is supported by the National Center for Advancing Translational Sciences of the National Institutes of Health Award Number UL1TR000114. We would like to thank the MDL and UMGC laboratories; U of MN for analyzing the Proseek Multiplex assays .

Main citations

Hankin JH, Stram DO, Arakawa K, et al. Singapore Chinese Health Study: Development, validation, and calibration of the quantitative food frequency questionnaire. Nutr Cancer 2001;39(2):187-95.

Kitahara CM, Trabert B, Katki HA, et al. Body mass index, physical activity, and serum markers of inflammation, immunity, and insulin resistance. Cancer Epidemiol Biomarkers Prev. 2014;23(12):2840-9.

Assarsson, E., Lundberg, M., Holmquist, G., Bjorkesten, J., Thorsen, S. B., Ekman, D., et al. (2014). Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PloS One, 9*(4), e95192. doi:10.1371/journal.pone.0095192 [doi]

Enroth, S., Johansson, A., Enroth, S. B., & Gyllensten, U. (2014). Strong effects of genetic and lifestyle factors on biomarker variation and use of personalized cutoffs. *Nature Communications, 5*, 4684. doi:10.1038/ncomms5684 [doi]