

Multiple Inflammatory Biomarker Detection in a Prospective Cohort Study: A Cross-Validation between Well-Established Single-Biomarker Techniques and an Electrochemiluminescence-Based Multi-Array Platform

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Abstract

Background: In terms of time, effort and quality, multiplex technology is an attractive alternative for well-established single-biomarker measurements in clinical studies. However, limited data comparing these methods are available.

Methods: We measured, in a large ongoing cohort study (n = 574), by means of both a 4-plex multi-array biomarker assay developed by MesoScaleDiscovery (MSD) and single-biomarker techniques (ELISA or immunoturbidimetric assay), the following biomarkers of low-grade inflammation: C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1). These measures were realigned by weighted Deming regression and compared across a wide spectrum of subjects' cardiovascular risk factors by ANOVA.

Results: Despite that both methods ranked individuals' levels of biomarkers very similarly (Pearson's $r \geq 0.755$) absolute concentrations of all biomarkers differed significantly between methods. Equations retrieved by the Deming regression enabled proper realignment of the data to overcome these differences, such that intra-class correlation coefficients were then 0.996 (CRP), 0.711 (SAA), 0.895 (sICAM-1) and 0.858 (sVCAM-1). Additionally, individual biomarkers differed across categories of glucose metabolism, weight, metabolic syndrome and smoking status to a similar extent by either method.

Conclusions: Multiple low-grade inflammatory biomarker data obtained by the 4-plex multi-array platform of MSD or by well-established single-biomarker methods are comparable after proper realignment of differences in absolute concentrations, and are equally associated with cardiovascular risk factors, regardless of such differences. Given its greater efficiency, the MSD platform is a potential tool for the quantification of multiple biomarkers of low-grade inflammation in large ongoing and future clinical studies.

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Introduction

Biomarker measurements representing low-grade inflammation have gained increasing importance in the management and understanding of cardiovascular disease (CVD) [1–8]. Low-grade inflammation is accompanied by inflammatory cells that closely

interact with the arterial wall, thereby driving the development of atherosclerosis and CVD [1,2]. Because the role of low-grade inflammation in the pathophysiology of CVD is multi-faceted [1,2], an extensive characterization by multiple biomarkers of low-grade inflammation is desirable. In this line, large cohort studies

are increasingly adopting such a multiple biomarker approach [8–15].

Among a large variety of potential biomarkers [16], acute-phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA), and vascular adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) reflect low-grade inflammation when present in low concentrations [1,2], whereas high concentrations, such as CRP > 10 mg/l, more likely reflect acute inflammation or infection [9,17,18]. These four biomarkers of low-grade inflammation were studied because they play an important role in the pathophysiology of CVD [1,2] and higher concentrations have been associated with (incident) CVD [3–8].

Traditionally, well-established analytical methods have enabled the analysis of single biomarkers of low-grade inflammation in one run. However, obtaining multiple biomarkers based on many single-biomarker measurements is very labor intensive, expensive and requires (relatively) large sample volumes. These limitations hamper an efficient multiple biomarker approach, particularly in large observational cohort or clinical trial studies. An attractive solution to these limitations is the simultaneous, and thus more efficient, measurement of a set of low-grade inflammatory biomarkers in one run. Such methods have recently become available with the use of multi-array platforms, such as the Luminex® and the MesoScaleDiscovery® (MSD) platforms and provide the tools necessary for efficient multiple biomarker detection. However, it remains to be established to what extent biomarker concentrations, as measured with these multi-array platforms, are comparable to well-established single-biomarker measurements. Although some cross-validation studies have been performed, most have not focused on biomarkers of low-grade inflammation [19–23] and the only study that did so pointed the problem of different measured concentrations, which may lead to bias in epidemiological associations [23].

Therefore, introducing a multi-array platform in the context of an ongoing longitudinal cohort study poses some challenges [24,25] and cross-validation between methods within such a cohort is necessary before the ‘new’ method may replace the ‘old’ one. Specifically, one needs to determine whether substantial differences in biomarker concentrations are introduced by the new method, in which case realignment of the data by appropriate mathematical transformations may be required for the investigation of within-subjects changes in absolute concentrations of biomarkers over the course of time [26,27]. In addition, the data obtained need also to be similarly associated with risk factors (RFs) known to be associated with low-grade inflammation to ensure that the multi-array platform measures what it intends to measure (i.e. face validity).

In view of these considerations, we compared the performance of a 4-plex multi-array electrochemiluminescence detection platform of low-grade inflammatory biomarkers (CRP, SAA, sICAM-1 and sVCAM-1) of MSD with that of well-established single-biomarker measurements, in a large ongoing cohort study of individuals with a wide spectrum of cardiovascular risk factors (RFs) known to be associated with low-grade inflammation.

Materials and Methods

Ethics Statement

The study was approved by the Medical Ethical Committee of the Maastricht University and all individuals gave written informed consent.

Study Population and Design

The Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) is a prospective cohort study that was originally designed to study the effects of obesity, glucose and lipid metabolism, lifestyle and genetics on cardiovascular complications, as described in detail elsewhere [28–33]. Briefly, individuals were selected from a large population-based cohort and included if they were of Caucasian ethnicity and older than 40 years, and met one or more of the following criteria: a body mass index (BMI) ≥ 25 kg/m², a positive family history for type 2 diabetes mellitus, a history of gestational diabetes, use of anti-hypertensive medication, a postprandial glucose ≥ 6.0 mmol/l and/or glucosuria. In total, 574 individuals [mean age 59.6 \pm 7.0 years; 38.7% women] were included and extensively characterized with regard to their metabolic, cardiovascular and lifestyle risk profiles during 2 visits to the University research unit (CODAM-1, baseline examination: September 1999–July 2002). A first follow-up examination took place among 495 individuals (14% drop-out rate, mainly due to morbidity or mortality) approximately 7 years later (CODAM-2, July 2006–November 2009).

At baseline (i.e. CODAM-1), biomarkers of low-grade inflammation were assessed by single-biomarker techniques. At follow-up (i.e. CODAM-2), the single-biomarker techniques were replaced by the multi-array platform of MSD. To ensure comparability between methods, biomarkers of low-grade inflammation were also reassayed by the multi-array platform of MSD in all samples from the baseline examination (i.e. CODAM-1); at the time of these measurements, baseline samples had thus been stored for ~ 7 years. The present cross-validation study reports on individuals’ paired data on biomarkers during the baseline examination (CODAM-1) and thus is a cross-sectional method comparison study. Method comparison for each biomarker was conducted on paired data, which were available for CPR in 566 individuals, for SAA in 563 individuals, for sICAM-1 in 566 individuals and for sVCAM-1 in 567 individuals and full paired data on all four inflammatory biomarkers were available in 550 individuals.

The CODAM study population is characterized by a wide spectrum of conditions known to be associated with low-grade inflammation [28–33]. Specifically, 52.4% of the individuals had normal glucose metabolism (NGM), 22.2% had impaired glucose metabolism (IGM) and 25.4% had type 2 diabetes mellitus (DM2) [28–33]. On the basis of measured BMI, the prevalence of normal weight, overweight and obesity was 18.2, 50.9 and 30.9%, respectively [32]. The metabolic syndrome, i.e. the clustering of ≥ 3 out of 5 cardiovascular RFs reflecting central obesity, dyslipidemia, elevated blood pressure and fasting plasma glucose, was prevalent in 54.7% of the study population [30,31]. On the basis of self-reports, 29.3% of the individuals were never-smokers, 50.5% were ex-smokers and 20.2% were current smokers [33]. The prevalence of CVD was 27.6% and based on self-reports of myocardial infarction, coronary bypass surgery, stent placement or balloon dilatation, transient ischemic attack or stroke, abnormalities on a 12-lead electrocardiogram (Minnesota codes 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3 or 7.1) and/or self-reported narrowing of peripheral arteries, non-traumatic limb amputation or a measured ankle-arm index < 0.9 [30,31]. Glycated hemoglobin [(mean \pm SD) 6.0 \pm 0.82%] was measured as previously described [28–33]. Estimated glomerular filtration rate (eGFR) [(mean \pm SD) 95.7 \pm 19.0 mL/min/1.73 m²] was calculated on the basis of individuals’ age, sex and serum creatinine levels according to the short Modification of Diet in Renal Disease (MDRD) equation [34].

Table 1. Absolute biomarker concentrations in the total population and according to glucose metabolism, weight, metabolic syndrome and smoking status as determined by the single-biomarker techniques or by the multi-array platform of MesoScaleDiscovery.

	CRP (mg/l)		SAA (mg/l)		sICAM-1 (µg/l)		sVCAM-1 (µg/l)	
	Immunoturbidimetry	Multi-array	ELISA	Multi-array	ELISA	Multi-array	ELISA	Multi-array
Total population (n = 550)	2.6 [1.4–4.5]	1.9 [0.9–3.8]	7.0 [4.0–13.8]	1.3 [0.8–2.2]	350±91	219±54	476±121	339±75
Glucose metabolism status^a								
NGM (n = 291)	2.2 [1.3–3.7]	1.6 [0.9–3.1]	6.2 [3.7–11.8]	1.2 [0.7–2.0]	338±85	210±50	463±121	331±73
IGM (n = 122)	2.8 [1.5–4.8]	2.1 [1.0–3.9]	8.0 [4.5–14.9]	1.3 [0.9–2.6]	354±90	220±48	467±105	338±68
DM2 (n = 137)	3.2 [1.9–5.7]	2.4 [1.3–5.3]	8.1 [4.7–15.5]	1.5 [1.0–2.6]	373±100	237±63	510±127	356±82
Weight status^b								
Normal weight (n = 100)	1.4 [0.9–3.0]	1.0 [0.5–2.4]	5.3 [3.0–12.0]	1.0 [0.6–1.9]	326±99	205±50	454±130	323±70
Overweight (n = 283)	2.2 [1.4–3.8]	1.6 [0.9–3.1]	6.4 [4.2–12.6]	1.2 [0.8–2.0]	341±79	214±47	467±114	334±73
Obese (n = 167)	3.6 [2.3–6.0]	3.0 [1.6–5.4]	8.6 [5.2–15.8]	1.6 [1.0–2.7]	380±98	238±62	505±121	357±76
Metabolic syndrome status^c								
0–1 risk factor (n = 134)	1.5 [0.9–3.1]	1.0 [0.5–2.4]	5.6 [3.4–11.6]	1.1 [0.7–1.8]	317±75	198±42	447±106	322±65
2 risk factors (n = 119)	2.4 [1.3–3.8]	1.8 [0.9–3.2]	6.5 [4.0–14.7]	1.3 [0.8–2.5]	334±80	205±41	464±117	324±67
≥3 risk factors ^d (n = 297)	3.0 [1.8–5.2]	2.4 [1.3–4.7]	7.7 [4.5–14.2]	1.4 [0.9–2.4]	372±96	235±58	494±126	352±79
Smoking status								
Never (n = 161)	2.1 [1.3–3.4]	1.5 [0.9–2.7]	6.1 [3.8–13.6]	1.3 [0.8–2.1]	331±82	211±49	485±125	337±75
Ex-smoker (n = 278)	2.6 [1.4–4.6]	2.0 [0.9–3.9]	6.8 [4.2–12.6]	1.3 [0.8–2.0]	347±88	217±57	481±117	344±73
Current (n = 111)	3.2 [1.5–5.5]	2.4 [1.0–5.3]	9.4 [4.5–15.6]	1.5 [0.9–2.7]	385±101	237±50	450±122	327±78

Data are means ± SD or medians [interquartile range].

^aNGM, normal glucose metabolism: defined as fasting plasma glucose <6.1 mmol/l and 2-hour post-load plasma glucose <7.8 mmol/l; IGM, impaired glucose metabolism: includes impaired fasting plasma glucose (between 6.1 mmol/l and 7.0 mmol/l) and/or impaired glucose tolerance (2-hour post-load plasma glucose between 7.8 and 11.1 mmol/l); DM2, diabetes mellitus type 2 (fasting plasma glucose ≥7.0 mmol/l and/or 2-hour post-load plasma glucose ≥11.1 mmol/l);

^bCategorized on the basis of individuals' body mass index (BMI) as: normal (if BMI 18.5–24.9 kg/m²); overweight (if BMI ≥25.0 and <29.9 kg/m²), and obese (if BMI ≥30 kg/m²);

^cMetabolic syndrome status was defined according to the revised NCEP-ATPIII definition (American Heart Association/National Heart, Lung and Blood Institute);

^dany 3 out of the following traits/risk factors reflect the presence of the syndrome: elevated waist circumference (≥102 cm in men, ≥88 cm in women); reduced HDL-cholesterol (<1.03 mmol/l in men, <1.29 mmol/l in women, and/or specific drug treatment); elevated triglycerides (≥1.7 mmol/l and/or specific drug treatment); elevated blood pressure (systolic/diastolic ≥130/85 mm Hg and/or anti-hypertensive treatment); and elevated fasting plasma glucose (≥5.6 mmol/l and/or glucose-lowering treatment); CRP, C-reactive protein; SAA, serum amyloid A; sICAM-1, soluble intercellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1.

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Biomarker Assessments

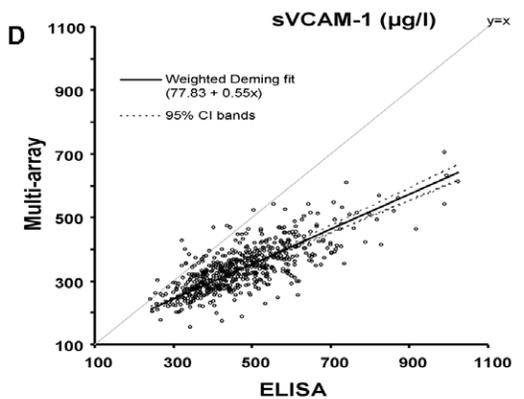
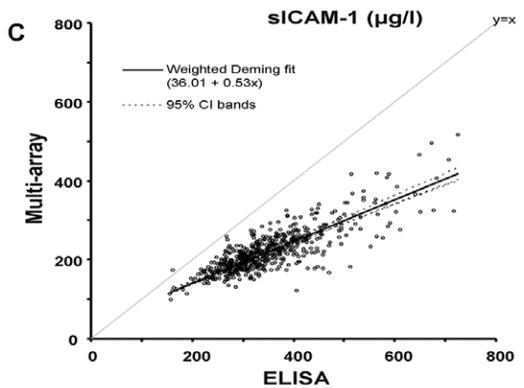
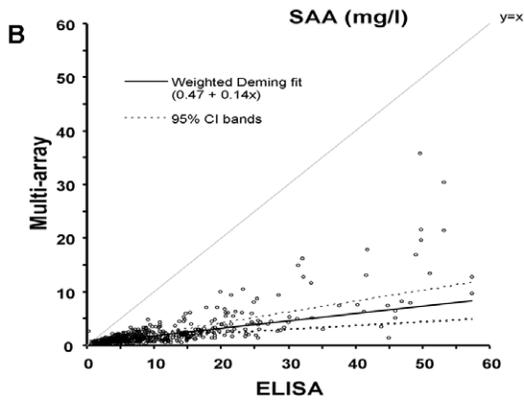
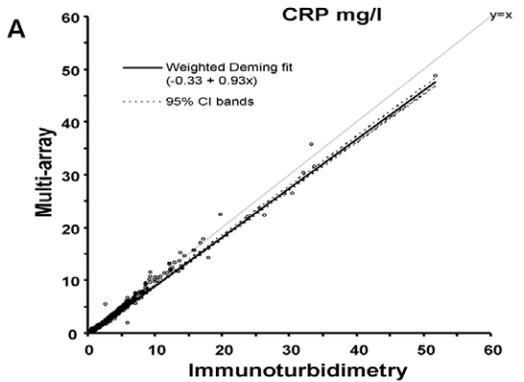
Individuals were asked to stop their lipid-lowering medication 14 days prior to the blood withdrawals and all other medication on the day before. After an overnight fast (duration of at least 10 hours) blood was drawn from the antecubital vein and collected in EDTA polypropylene tubes for plasma and in clot activator containing polypropylene tubes for serum. EDTA tubes were centrifuged at 3000 rpm for 15 min at 4°C, and plasma was immediately divided into 1 ml aliquots and stored in –80°C freezers until further analysis. Tubes with clot activator were left 20 minutes before centrifugation at 3000 rpm for 15 min at 20°C, and serum was immediately divided into 1 ml aliquots and stored in –20°C freezers until analysis [28].

Biomarker detection by single-biomarker techniques. CRP was measured in a single measurement in serum with a high-sensitivity, immunoturbidimetric assay (detection range 100 ng/ml to 20000 ng/ml, i.e. factor 200) (Latex, Roche Diagnostics Netherlands BV, Almere, The Netherlands, www.roche.nl). This assay is based on the principle of particle-enhanced immunological agglutination. Briefly, anti-CRP antibodies coupled to latex micro-particles react with antigen present in the sample to form antigen-antibody complexes. Then, these micro-particles with antigen-antibody complexes agglutinate. This

changes the fluid turbidity of the sample, which is detected by turbidimetry. sVCAM-1 was measured in EDTA plasma with a high-sensitivity human Quantikine ELISA kit (detection range 6.25 ng/ml to 200 ng/ml, i.e. factor 32) (R&D Systems, Minneapolis, MN, USA, www.rndsystems.com). sICAM-1 (detection range 0.625 ng/ml to 10 ng/ml, i.e. factor 16) and SAA (detection range 9.4 ng/ml to 600 ng/ml, i.e. factor 64) were measured in EDTA plasma by ELISA (Biosource, Invitrogen, Carlsbad, CA, USA, www.invitrogen.com). All low-grade inflammatory biomarkers were measured at the Laboratory of Toxicology, Genetics and Pathology of the National Institute for Public Health and the Environment, Bilthoven, The Netherlands [30]. The intra- and inter-assay coefficients of variation (CVs) for these assays were, for CRP, 0.6% and 1.9%; for SAA, 6.1% and 17.5%; for sICAM-1, 5.6% and 6.6%; and for sVCAM-1, 3.1% and 4.7%, respectively.

Biomarker detection by the 4-plex multi-array electrochemiluminescence detection platform of MesoScaleDiscovery. The 4-plex multi-array electrochemiluminescence platform of MesoScaleDiscovery (detection range 0.008 ng/ml to 1000 ng/ml, i.e. factor 125000) (MesoScaleDiscovery, Gaithersburg, MD, USA, www.mesoscale.com) was used to measure the four low-grade inflammatory biomarkers (CRP,

Weighted Deming Regression



Bland-Altman plots

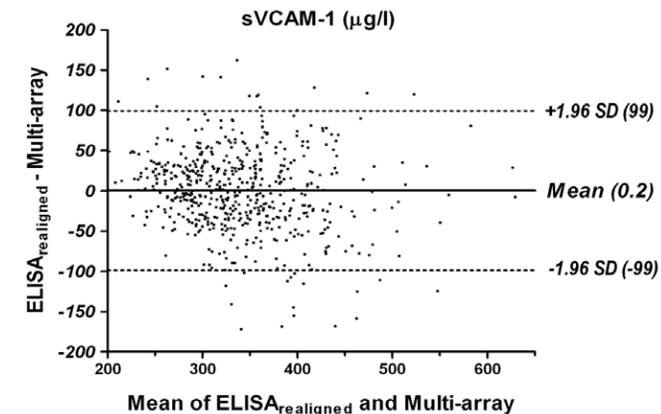
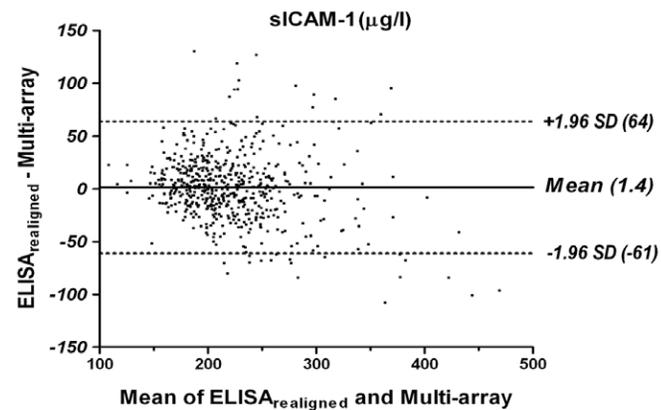
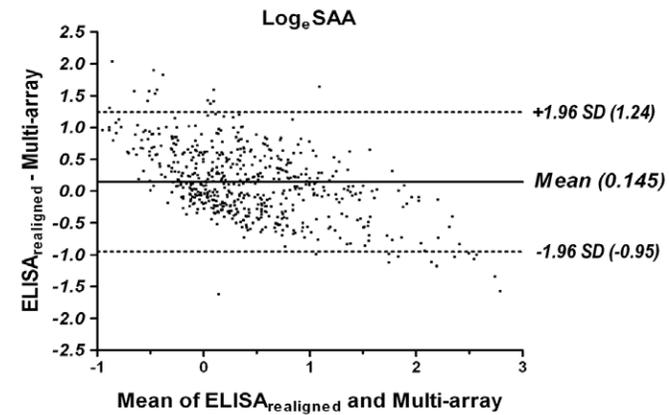
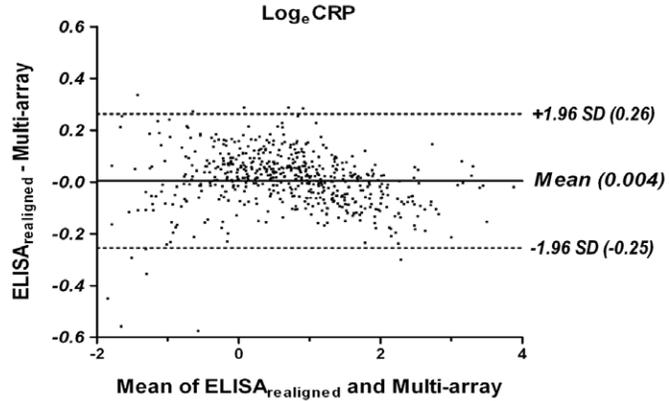


Figure 1. Left panel: comparison of biomarker concentrations between the single-biomarker techniques and the multi-array platform by weighted Deming regression; Right panel: Bland-Altman plots of the differences between the two methods. (A) CRP, C-reactive protein (n=566); (B) SAA, serum amyloid A (n=563); (C) sICAM-1, soluble intercellular adhesion molecule 1 (n=566); and (D) sVCAM-1, soluble vascular cell adhesion molecule 1 (n=567). For \log_e transformed data limits of agreement can be converted to original units by anti-log calculation (e.g., for CRP, $e^{-0.25}$ and $e^{0.26}$ which are equivalent to 0.78 and 1.30 times or 22% below or 30% above the long axis of CRP, respectively).
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SAA, sICAM-1 and sVCAM-1) simultaneously in EDTA plasma. This system uses multi-array plates fitted with multi-electrodes per well with each electrode being coated with a different capture antibody. For the present study the 4-plex assay (plates fitted with four electrodes per well, i.e. four separate well spots with a different capture antibody bound to each) was used. The assay procedure follows that of a classic sandwich ELISA with any of the

analytes of interest captured on the relevant electrode. These captured analytes were, in turn, detected by a secondary, analyte-specific, ruthenium-conjugated antibody, which is capable of emitting light after electrochemical stimulation. This method minimizes nonspecific signals as the stimulation mechanism (electricity) is decoupled from the signal (light). According to the MSD protocol, each sample was analyzed in duplicate on the same

Box-and-whisker plots

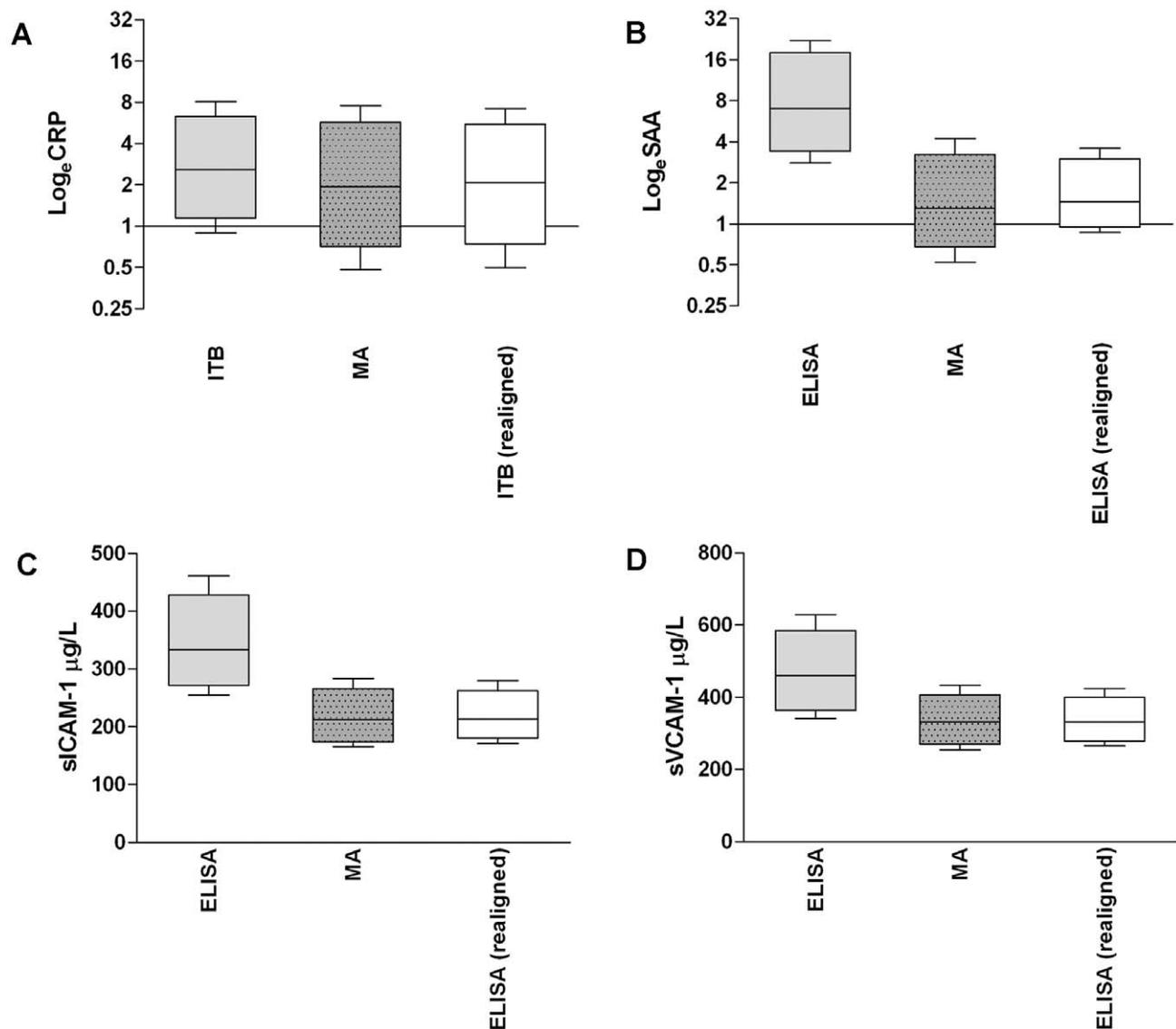


Figure 2. Box-and-whisker plots showing median and interquartile (box) and 10th and 90th centiles (whiskers) for each biomarker, before and after realignment to current (multi-array) concentrations. (A) CRP, C-reactive protein (n=566); (B) SAA, serum amyloid A (n=563); (C) sICAM-1, soluble intercellular adhesion molecule 1 (n=566); and (D) sVCAM-1, soluble vascular cell adhesion molecule 1 (n=567); ITB, immunoturbidimetry; MA, multi-array.
doi:10.1371/journal.pone.0058576.g002

Table 2. Weighted Deming regression.

Variable	N pairs	Regression equations (weighted Deming) ^a						
		Y	X	intercept		slope		Sylx
				α	95% CI	β	95% CI	
CRP (mg/l)	566	CRP _{MA}	CRP _{ITB}	-0.33	-0.35; -0.31	0.93	0.91; 0.94	0.102
SAA (mg/l)	563	SAA _{MA}	SAA _{ELISA}	0.47	0.05; 0.90	0.14	0.07; 0.20	0.239
sICAM-1 (μ g/l)	566	sICAM-1 _{MA}	sICAM-1 _{ELISA}	36.01	24.63; 47.39	0.53	0.49; 0.56	0.104
sVCAM-1 (μ g/l)	567	sVCAM-1 _{MA}	sVCAM-1 _{ELISA}	77.83	58.90; 96.75	0.55	0.51; 0.59	0.124
CRP (mg/l)	566	CRP _{ITB}	CRP _{MA}	0.35	0.34; 0.37	1.08	1.06; 1.10	0.110
SAA (mg/l)	563	SAA _{ELISA}	SAA _{MA}	-3.46	-7.37; 0.45	7.33	4.40; 10.25	1.751
sICAM-1 (μ g/l)	566	sICAM-1 _{ELISA}	sICAM-1 _{MA}	-68.49	-94.71; -42.27	1.90	1.77; 2.03	0.197
sVCAM-1 (μ g/l)	567	sVCAM-1 _{ELISA}	sVCAM-1 _{MA}	-141.52	-186.54; -96.49	1.82	1.68; 1.96	0.225

^aData are intercepts (α) and (slopes) β of the Deming regression equation, which all differed significantly from 0 and 1, respectively, as indicated by their 95% CI; rejection of the hypothesis that $\alpha=0$ means that the two methods differ at least by a constant amount; rejection of the hypothesis that $\beta=1$ implies that there is a proportional difference between methods; Sylx are standard deviations of the residuals;

Upper panel: these equations are used as cross-validation equations to realign single-biomarker data (ITB, immunoturbidimetry; or ELISA) to multi-array (MA) data. Lower panel: these equations are used as cross-validation equations to realign multi-array (MA) data to single-biomarker data (ITB, immunoturbidimetry; or ELISA). CRP, C-reactive protein; SAA, serum amyloid A; sICAM-1, soluble intercellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1.

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array plate. All multi-array plates were analyzed within 16 days. The intra- and inter-assay CVs for the platform of MSD were, for CRP, 3.0% and 4.1%; for SAA, 2.5% and 11.8%; for sICAM-1, 2.5% and 4.7%; and, for sVCAM-1, 2.6% and 5.0%, respectively.

Variation between production lots of multi-array plates could influence biomarker measurements. We have evaluated the possible effect of lot-to-lot variation in the current 4-plex assay using additional data of previous studies [35,36]. Based on biomarker data of 6 separate production lots (with an average of 30 plates per lot) the lot-to-lot CV for CRP was 9.8%, for SAA was 28.9%, for sICAM-1 was 3.4% and for sVCAM-1 was 4.9%. Thus, these variations were quite acceptable, except for SAA. Still, to avoid any noise due to lot-to-lot variation, all plasma samples of the CODAM study were measured within a single production lot of multi-array plates.

Statistical Analyses

Method comparisons. Absolute concentrations of each biomarker as measured by the single-biomarker techniques and the multi-array platform were examined on all paired samples from the CODAM study baseline examination ($n = 566$ for CRP, $n = 563$ for SAA, $n = 566$ for sICAM-1 and $n = 567$ for sVCAM-1, after exclusion of erroneous outliers [37]). Pearson's correlation coefficients were used to assess whether the ranking of each biomarker was similar between methods. Weighted Deming regression was used to assess the extent of constant and/or proportional bias between methods [26,27]. This state-of-the-art statistical technique for method comparison is superior to simple linear regression by taking into account the error in both the dependent and independent variables [37,38]. In addition, it allows random errors of each method to be proportional to the measured concentrations, such that the ratio of the CVs between methods remains constant over the concentration ranges (set at 1:1 for regression calculations; e.g. 2% vs. 2% at low ranges, and 10% vs. 10% at high ranges) [37,38].

Realignment and agreement. We anticipated that absolute biomarker concentrations, as obtained by either single- or multi-array methods, would differ due to a lack of standardization. Realignment of the data would, therefore, be necessary to enable

direct comparison of absolute concentrations. For that purpose we used equations derived from Deming regression analyses to realign the data as obtained by one to the other method.

To examine the levels of agreement and verify the absence of systematic error *after* the re-alignment procedure, Bland-Altman plots of the differences between single-biomarker and multi-array data *vs.* their mean were obtained [39]. Bland-Altman plots were drawn on log₁₀ transformed data whenever the distribution of the differences was skewed [39,40]. In addition, two-way mixed effects models (absolute agreement) were used to calculate intra-class correlation coefficients (ICC), which reflect similarity in individuals' rank and similarity in absolute biomarker concentrations as obtained by single-biomarker techniques (*realigned*) and multi-array platform [41]. Note that the results of these analyses are shown in detail for single-biomarker data realigned to multi-array data for the following reason. The multi-array platform has recently been introduced in the CODAM study population and represents the methodology intended to carry on in follow-up assessments in this cohort.

Method performance across different cardiovascular risk groups. We used ANOVA to investigate the extent to which biomarker concentrations, either assessed by the single-biomarker techniques or the multi-array platform, increased across categories of glucose metabolism (i.e. NGM, IGM and DM2), weight (i.e. normal weight, overweight and obesity), number of traits of the metabolic syndrome (0–1 RFs, 2 RFs and ≥ 3 RFs) and smoking status (never, ex- and current-smoker), by appreciation of the group effects. ANOVA for repeated measures were subsequently used to ascertain whether such patterns of associations were similar between methods, by appreciation of group-by-method effects (the P-values of which should then be ≥ 0.05). In these analyses, (non-aligned) individual biomarker data, which are expressed in different scale units, were first standardized to comparable units by calculation of Z-scores as follows: (the individuals' value – the population mean) / the population SD. Per definition, each Z-score has a mean of 0, a SD of 1, and the same distribution as the absolute biomarker concentration (i.e. the ranking of individuals in the population remains the same). This thus enabled a direct comparison of the magnitude of relative

Table 3. Comparison of individual biomarker Z-scores according to glucose metabolism, weight, metabolic syndrome and smoking status as determined by the single-biomarker techniques or by the multi-array platform of MesoScaleDiscovery[®].

	Log _e CRP			Log _e SAA			sICAM-1			sVCAM-1		
	ITB	Multi-array	ELISA	Multi-array	ELISA	mean 95% CI	Multi-array	ELISA	mean 95% CI	Multi-array	ELISA	mean 95% CI
Glucose metabolism status												
NGM (n = 291)	-0.13	-0.24; -0.01	-0.13 -0.24; -0.02	-0.11 -0.23; 0.00	-0.09 -0.20; 0.02	-0.11 -0.23; 0.00	-0.14 -0.26; -0.03	-0.09 -0.20; 0.02	-0.08 -0.20; 0.03			
IGM (n = 122)	0.06	-0.12; 0.23	0.06 -0.11; 0.24	0.11 -0.06; 0.28	0.001 -0.17; 0.17	0.05 -0.13; 0.22	0.01 -0.16; 0.19	-0.09 -0.26; 0.09	-0.02 -0.19; 0.15			
DM2 (n = 137)	0.21	0.05; 0.38	0.22 0.05; 0.39	0.14 -0.02; 0.31	0.20 0.03; 0.36	0.20 0.03; 0.37	0.29 0.13; 0.46	0.26 0.10; 0.42	0.20 0.03; 0.36			
P-value for linear trend	0.001		0.014	0.005	0.003	0.003	<0.001	0.001	0.007			
P-value interaction group*method	0.655		0.146		0.152			0.357				
Weight status												
Normal weight (n = 100)	-0.53	-0.71; -0.34	-0.55 -0.74; -0.37	-0.28 -0.47; -0.09	-0.28 -0.47; -0.09	-0.24 -0.44; -0.05	-0.25 -0.44; -0.06	-0.17 -0.36; 0.02	-0.20 -0.39; -0.006			
Overweight (n = 283)	-0.06	-0.17; 0.05	-0.05 -0.16; 0.06	-0.04 -0.15; 0.07	-0.07 -0.18; 0.04	-0.09 -0.20; 0.03	-0.09 -0.20; 0.02	-0.09 -0.20; 0.03	-0.07 -0.19; 0.04			
Obese (n = 167)	0.41	0.27; 0.56	0.42 0.28; 0.56	0.23 0.09; 0.38	0.28 0.14; 0.43	0.29 0.14; 0.44	0.30 0.15; 0.45	0.24 0.10; 0.39	0.24 0.10; 0.39			
P-value for linear trend	<0.001		<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001			
P-value interaction group*method	0.185		0.567		0.952			0.881				
Metabolic syndrome status												
0-1 risk factors (n = 134)	-0.39	-0.56; -0.23	-0.43 -0.59; -0.26	-0.17 -0.34; 0.00	-0.16 -0.32; 0.01	-0.33 -0.49; -0.16	-0.36 -0.52; -0.20	-0.19 -0.36; -0.03	-0.18 -0.34; -0.01			
2 risk factors (n = 119)	-0.09	-0.26; 0.08	-0.09 -0.26; 0.08	-0.02 -0.19; 0.16	-0.002 -0.18; 0.17	-0.18 -0.35; -0.001	-0.26 -0.43; -0.09	-0.12 -0.29; 0.05	-0.21 -0.38; -0.03			
≥3 risk factors (n = 297)	0.21	0.10; 0.32	0.23 0.12; 0.34	0.08 -0.03; 0.20	0.07 -0.04; 0.18	0.22 0.11; 0.33	0.27 0.16; 0.38	0.14 0.02; 0.25	0.16 0.05; 0.27			
P-value for linear trend	<0.001		<0.001	0.016	0.028	<0.001	<0.001	0.001	0.001			
P-value interaction group*method	0.002		0.922		0.103			0.322				
Smoking status												
Never (n = 161)	-0.23	-0.39; -0.08	-0.24 -0.40; -0.09	-0.17 -0.33; -0.02	-0.14 -0.29; 0.02	-0.22 -0.38; -0.07	-0.18 -0.34; -0.03	0.07 -0.08; 0.22	-0.03 -0.18; 0.13			
Ex-smoker (n = 278)	0.07	-0.05; 0.18	0.06 -0.06; 0.18	0.03 -0.08; 0.15	-0.001 -0.12; 0.11	-0.02 -0.14; 0.10	-0.03 -0.14; 0.09	0.01 -0.10; 0.13	0.05 -0.07; 0.16			
Current (n = 111)	0.18	-0.01; 0.36	0.20 0.01; 0.38	0.17 -0.02; 0.35	0.20 0.02; 0.38	0.38 0.19; 0.56	0.33 0.14; 0.51	-0.13 -0.31; 0.05	-0.08 -0.26; 0.11			
P-value for linear trend	0.001		<0.001	0.006	0.007	<0.001	<0.001	0.101	0.687			
P-value interaction group*method	0.205		0.543		0.489			0.144				

Data are means of standardized biomarker concentrations (Z-scores); all data are adjusted for sex, age, estimated glomerular filtration rate and prior CVD; NGM, normal glucose metabolism; IGM, impaired glucose metabolism; DM2, diabetes mellitus type 2; CRP, C-reactive protein; SAA, serum amyloid A; sICAM-1, soluble intercellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; ITB, immunoturbidimetry.
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Table 4. Agreement in risk level assignment on the basis of CRP obtained by immunoturbidimetry and the multi-array platform.

CRP	Level	Multi-array platform					Multi-array platform realigned to Immunoturbidimetry					
		<1 mg/l (%)	1–3 mg/l (%)	>3 mg/l (%)	Total (%)	κ	Concordance (%)	<1 mg/l (%)	1–3 mg/l (%)	>3 mg/l (%)	Total (%)	Concordance (%)
Immunoturbidimetry	<1 mg/l	12.9	0.0	0.0	12.9	0.641	12.5	0.4	0.0	12.9	0.946	0.946
	1–3 mg/l	15.1	31.3	0.0	46.4		0.5	45.5	0.4	46.4		
	>3 mg/l	0.0	8.2	32.5	40.7		0.0	2.0	38.7	40.7		
	Total	28.0	39.5	32.5	100.0	76.7	13.1	47.8	39.1	100.0	96.7	
Immunoturbidimetry realigned to multi-array	<1 mg/l	26.0	0.4	0.0	26.4	0.931	-	-	-	-	-	-
	1–3 mg/l	2.0	37.6	0.7	40.4		-	-	-	-		
	>3 mg/l	0.0	1.5	31.8	33.3		-	-	-	-		
	Total	28.0	39.5	32.5	100.0	95.4	-	-	-	-	-	-

κ, Cohen's kappa (measure of agreement for categorical data); –, not applicable. doi:10.1371/journal.pone.0058576.t004

differences in each biomarker by RF categories. All comparisons included adjustments for sex, age, eGFR and prior CVD and were conducted among individuals with complete paired data on all four biomarkers (n = 550).

All analyses were performed with the use of the Statistical Package for Social Sciences (SPSS Inc, version 15.0, Chicago, Illinois, USA, www.spss.com), except weighted Deming regression, which was analyzed using the Analyse-It software (Analyse-it Software Ltd, Leeds, UK, www.analyse-it.com) for Microsoft Excel (Microsoft Corporation, Washington, USA, www.microsoft.com). Statistical significance was set at a P-value <0.05.

Results

Biomarker Concentrations

Table 1 shows the absolute concentrations of CRP, SAA, sICAM-1, and sVCAM-1, as measured with the single-biomarker techniques or the multi-array platform, in the whole study population and across RFs categories.

Method comparison. Despite the very high Pearson's correlation coefficients (i.e. 0.994 for CRP, 0.758 for SAA, 0.816 for sICAM-1 and 0.755 for sVCAM-1) absolute concentrations of biomarkers as obtained by single-biomarker vs. multi-array techniques differed considerably. Indeed, weighted Deming regression analyses for all biomarkers showed significant constant (intercepts) and proportional (slopes) bias between methods such that the absolute mean concentrations of all four biomarkers were lower when measured with the multi-array platform than with the single-biomarker techniques (Fig. 1A–D, left panels). The above indicates that, when comparing *absolute* values, realignment of the single-biomarker data to the multi-array data (or vice-versa) is thus warranted.

Realignment and agreement. Realignment of the data as obtained by different methods was therefore conducted with the use of the coefficients retrieved from the Deming regression models (Table 2). Bland-Altman plots of the single-biomarker data *realigned* to the multi-array data (Fig. 1A–D, right panels) showed that no obvious relation of differences between methods with their mean was present. For all biomarkers, except SAA, Bland-Altman plots confirmed the removal of systematic bias after the realignment (all mean values for differences between methods around 0 (Fig. 1A, 1C and 1D, right panels)). For SAA, a systematic difference between ELISA and multi-array data still persisted after the realignment (about 15%, i.e. $e^{0.145}$ as compared to their mean (Fig. 1B, right panel)). In addition, the equations applied for the *realignment* (Table 2) resulted in similar distributions of single-biomarker and multi-array data (Fig. 2). The resulting ICCs between single-biomarker (*realigned*) and multi-array data were 0.996 for CRP, 0.711 for SAA, 0.895 for sICAM-1 and 0.858 for sVCAM-1.

Method performance across different cardiovascular risk groups. Concentrations of all biomarkers, as measured by single-biomarker or multi-array methods (expressed as Z-scores), increased significantly across categories of glucose metabolism, weight, metabolic syndrome and smoking status (all P-trends ≤ 0.028 , except for sVCAM-1 and smoking status), independently of sex, age, eGFR and prior CVD (Table 3). Importantly, the patterns of associations between RFs level and individual biomarker concentrations did not differ by method of detection [all P-values for group*method interaction > 0.05 , except for metabolic syndrome status and Log_eCRP (P-value = 0.002)] (Table 3).

These results did not materially change, when the analyses were repeated excluding individuals with CRP values >10 mg/l, likely

to indicate an acute inflammatory response [9,17,18] (data not shown).

Additional Analyses

A key step in biochemical tests comparison is to ascertain whether the level of agreement between methods is acceptable from a clinical standpoint [40]. For CRP, values <1, 1–3, and >3 mg/l have been proposed to identify individuals at low, intermediate and high-risk for incident CVD, respectively, whereas such values are lacking for the other biomarkers examined herein. This impairs the appreciation of the clinical relevance of the limits of agreement between methods obtained for these biomarkers (Fig. 1B–D, right panels) [8,9]. Still, for CRP we could ascertain that, on the basis of immunoturbidimetry, 12.9% of the CODAM study population would be classified as ‘low risk’, 46.4% at ‘intermediate risk’ and 40.7% at ‘high-risk’; on the basis of the multi-array platform these numbers would be 28.0%, 39.5% and 32.5%, respectively (Cohen’s $\kappa = 0.641$, which is a measure of agreement for categorical data; overall concordance rate of 76.7%). After realignment of the immunoturbidimetry to the multi-array data and vice versa, the agreement between methods increased considerably (Cohen’s κ of 0.931 and 0.946 and concordance rates of 95.4 and 96.7%, respectively - Table 4).

Discussion

The present study has three main findings. First, the absolute concentrations of CRP, SAA, sICAM-1 and sVCAM-1 differed significantly between the single-biomarker techniques and the multi-array platform of MSD. Second, equations retrieved by weighted Deming regression enabled proper realignment of the data to overcome these absolute differences. Finally, the overall pattern of associations between levels of the individual biomarkers with glucose metabolism, weight, metabolic syndrome and smoking status did not differ by method of detection. This is the first study that has examined and cross-validated, in a large ongoing cohort study, measurements of biomarkers of low-grade inflammation by means of single-biomarker techniques and the multi-array platform of MSD.

Our results are in line with a previous study, which suggested that data measured with single-biomarker techniques and data measured with the multi-array platform cannot be combined without appropriate realignment of the data as this would distort epidemiological associations [23]. In our study, the absolute concentrations of all four biomarkers were lower when measured with the multi-array platform than with the single-biomarker techniques. It should be emphasized, however, that the absolute concentration of each biomarker is based on the standards provided by the commercial kits and the lack of international standardization among these may therefore explain the differences between methods [9]. Although CRP reference materials exist, bias attributed to standardization remains due to the fact that reference materials were developed to distinguish between CRP values below 10 mg/l, from 10 to 40 mg/l and above 40 mg/l, whereas current assays aim for accurate and reproducible detection down to 0.3 mg/l [18]. Also according to the Centers for Disease Control and Prevention and the American Heart Association laboratory science discussion group, further standardization efforts are therefore required as measurements of absolute biomarker concentrations are of paramount importance for direct comparison between studies using different methods and for definition of clinical cutoff values [18]. Nevertheless, in the present study we were able to appropriately realign the data to overcome the absolute differences between both methods. Thus, the

introduction of a multi-array platform in an ongoing cohort study may be implemented without impairing the investigation of within-subject changes in biomarker concentrations over the course of time. This was enabled by re-assaying all the baseline samples with the new multi-array method. In addition, we show that the agreement in risk level assignment on the basis of CRP levels (<1, 1–3, and >3 mg/l [8,9]) is very high after realignment. It remains, however, that subjects’ risk-level assignment depends on the method used for CRP assessment, and that if this were done on the basis of MSD readings, less individuals from the CODAM Study would be considered to be at high-risk than if this were done on the basis of immunoturbidimetry readings. However, to establish which method is superior in risk prediction further studies are warranted.

Another option to directly compare individual biomarker levels between methods (but also between clinical studies) is by transformation of data to Z-scores, especially if realignment equations are lacking. By Z-score transformation, between-subjects ranking in terms of their biomarkers levels are preserved within the population. The present study shows that Z-scores of CRP, SAA, sICAM-1, sVCAM-1 differed across categories of glucose metabolism, weight, metabolic syndrome and smoking status in a similar fashion irrespective of the method of detection. Although it is evident that a high correlation between assays will result in identical associations, these results, illustrate and emphasize that, despite absolute differences, the relative differences are comparable between the single-biomarker techniques and the multi-array platform.

Taken together, our findings suggest that the multi-array platform of MSD could potentially replace the single-biomarker techniques for the detection of multiple biomarkers in large ongoing and future clinical studies aiming at the investigation of the role of low-grade inflammation in the etiology of CVD, though careful validation would be required.

Furthermore, the multi-array platform of MSD has several practical advantages over the well-established single-biomarker techniques for biomarker detection, although CRP assays are generally automated [18]: 1) it has simple operating procedures; 2) it has a higher sensitivity and greater detection range, which eliminates multiple dilutions and freeze and thaw cycles per sample; 3) it allows determination of four (or more) biomarkers simultaneously, improving the labor-efficiency, and due costs; and 4) it uses a small sample volume (5 μ L instead of 50 μ L for the detection of these four markers), which is useful in clinical and epidemiological studies.

The present study has some limitations. First, with the single-biomarker techniques, CRP was measured in serum and SAA, sVCAM-1 and sICAM-1 were measured in plasma, whereas with the multi-array platform all biomarkers were measured in plasma. This may, in part, explain the differences between methods in absolute concentrations of CRP, since a different matrix might effect detection. Furthermore, the measurement of biomarkers by the single-biomarker techniques and the multi-array platform were performed \sim 7 years apart, which could also have contributed to an underestimation of absolute biomarker concentrations by the multi-array platform. However, because storage time of samples was the same for all study individuals, if anything: 1) this underestimation was likely systematic and properly incorporated in the realignment equations; and 2) could not have affected the relative differences in biomarkers across different levels of subjects’ cardiovascular RFs. Second, we showed realignment equations to enable transition of ‘old’ to ‘new’ methods within our ongoing cohort study (and vice versa). However, the results were shown in detail for single-biomarker data realigned to multi-array data. This

way of presentation facilitates future comparisons of those biomarkers measured with the multi-array platform at follow-up examinations within this ongoing cohort study. However, any other cohort study should calculate realignment equations within their own data. These may be susceptible to lot-to-lot variation, although in our laboratory the lot-to-lot variation between multi-array assays was low for most of the biomarkers. Nevertheless, the measured concentrations will always depend on the standards provided by the commercial kits (for both the single biomarker and multi-array techniques), which have not been satisfactorily standardized internationally [9,18].

In conclusion, multiple biomarker detection by the 4-plex multi-array platform of MSD including CRP, SAA, sICAM-1 and sVCAM-1 shows comparable results with well-established single-biomarker techniques, despite differences in absolute concentrations. Subjects' risk-level assignment therefore depends on the method used. It is, however, uncertain which method is superior in risk prediction. Nevertheless, these biomarkers of low-grade

inflammation are associated with glucose metabolism, weight, metabolic syndrome and smoking status, irrespective of the method of detection. In terms of time, effort and quality, this multi-array platform of MSD is an attractive alternative for single-biomarker measurements. Therefore, this platform is a potential tool for the quantification of multiple biomarkers of low-grade inflammation using small sample volume in one single run in large ongoing and future clinical studies.

Author Contributions

Critically revised the manuscript: MPHvdW MMJvG CJHvdK RMAH EJMF CDAS. Read and approved the manuscript: BCTvB IF MPHvdW MMJvG CJHvdK RMAH EJMF CDAS CGS. Conceived and designed the experiments: IF MMJvG CJHvdK RMAH EJMF CDAS CGS. Performed the experiments: BCTvB IF MPHvdW. Analyzed the data: BCTvB IF MPHvdW. Contributed reagents/materials/analysis tools: IF MMJvG CJHvdK RMAH EJMF CDAS CGS. Wrote the paper: BCTvB IF CGS.

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