Original article

Adult aggressive behavior in humans and biomarkers: a focus on lipids and methylation

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Abstract

Aggression shows large variation between individuals, with about 50% explained by genetic factors. Biomarkers related to aggression have been reported for lipid metabolism and for epigenetic marks. Methylation and blood lipid levels are not independent and differential methylation can be a consequence of variation in blood lipid levels. We hypothesized that the methylation level of such loci in blood can inform us if aggression is associated with long-term exposure to lipid levels. If this is the case, we expect to find that loci where methylation levels are influenced by lipid levels to show differential methylation in aggressive individuals. Such loci might complement classic lipid level measures as a biomarker for lipid-related disturbances in aggression. As a first step, we examined the association of lipid levels and related biomarkers with aggression in a large adult population cohort (N = 5,588) and in 31 monozygotic (MZ) twin pairs who were discordant for aggression, as well as 12 extremely discordant MZ pairs. Biomarkers were not significantly associated with aggression in the population cohort. In the discordant MZ pairs we identified significant within-pair differences for glucose and marginally significant differences for lipids and cytokines, with the more aggressive twin showing lower levels of glucose and low density lipoprotein cholesterol and higher levels of fibrinogen, C-reactive protein and interleukin-6. The analysis of epigenetic data in the MZ pairs discordant for aggression did not show enrichment for lipid cytosine guanine dinucleotides (CpGs) and we observed no enrichment of lipid CpGs in an epigenome-wide association study of aggression in the population cohort. These results did not support the hypothesis that lipid CpGs show differential methylation in

adult aggression. A next step will be to examine the role of biomarkers in aggression across the lifespan, including childhood, and to explore a more holistic biomarker approach, such as offered by metabolomics.

Keywords

Adult aggression, lipids, epigenetics, biomarkers, discordant twin pairs.

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Introduction

Interpersonal violence and aggression place an enormous burden on society. While the incidence rate of injuries due to interpersonal violence has globally declined by approximately 11% from 1990 to 2013 and the disability-adjusted life year (DALY) rate has globally decreased by approximately 19.1%, the number of years lived with aggression-caused disability (YLD) is still high (12 YLD) [1]. In addition to the physical injuries sustained through violence, exposure to violence has negative health consequences throughout the lifespan, resulting in mental health and behavioral disorders and chronic diseases [2, 3]. Mental health problems are not only a consequence of exposure to violence. Inappropriate aggressive behavior is also part of a number of psychiatric disorders, such as conduct (CD) and oppositional defiant disorders (ODD), schizophrenia and substance use disorders [4] and a distinct feature of several single-gene human diseases such as glass syndrome [5].

Aggressive and antisocial behaviors are not limited to adults. In fact, physical aggression in preschool children is common, with a peak of aggressive behavior around age two. A steady decline is observed once children reach school age, with aggression continuing to decrease into early adulthood [6, 7]. A small group of children will continue to display aggressive behavior from childhood into adolescence and adulthood [8]. Twin-family studies have shown that both genetic and environmental factors play a role in the longitudinal stability of aggressive behavior from childhood to adolescence [9-11]. Across all ages and aggression-related problems, genetic factors account for approximately half of the phenotypic variance, with the other half due to environmental influences [12].

Epigenetic mechanisms may mediate the effects of the environment and the genome on complex traits by influencing the activity of gene expression. In humans, epigenetic processes are mostly studied by DNA methylation of candidate genes or across the entire genome. In most human tissues, DNA methylation occurs almost exclusively at cytosine guanine dinucleotides (CpGs). CpG sites tend to cluster in so-called CpG-islands, which in humans are often found in the promotor regions of genes [13, 14]. DNA methylation changes during development and in response to the environment [13-15]. Previous candidate gene studies have focused on the methylation of genes involved in neurotransmitter or hormone regulation and genes encoding cytokines and their transcription factors. Hyper-methylation of promotor regions have been observed for the serotonin receptor gene (SLC6A4) in adults with childhood-limited physical aggression [16], for the oxytocin receptor gene (OXTR) in 4-16 year old boys with ODD and CD with increased callous-unemotional (CU) traits [17] and for the monoamine oxidase A gene (MAO-A) in incarcerated adult males with antisocial personality disorder (APD) [18]. In contrast, hypo-methylation of the promotor has been observed for the glucocorticoid receptor gene (NR3C1) in young adults with lifetime externalizing problems [19] and for the serotonin 1B receptor gene (HTR1B) for 3-16 year old boys with increased CU traits [20]. Forty-eight genomic loci for interleukin-1α, 4, 6, 8 and 10 and 66 loci within the transcriptional regulators of these cytokines were differentially methylated in young adult males with childhood physical aggression compared to matched controls [21]. Genome-wide methylation studies in T-cells found 744 differentially methylated CpG sites in males and females with childhood physical aggression [22, 23], for 7 loci the cord blood was differentially methylated between children with early-onset CD problems and children with low CD

problems [24]. An epigenome-wide association study (EWAS) of adult aggression observed suggestive differential methylation for eight CpG sites and identified three additional suggestive markers in monozygotic (MZ) twins who were discordant for aggression [25]. These studies demonstrated an association between DNA methylation and aggression at various loci. It remains, however, unclear which of these associations reflect a causal effect of DNA methylation on aggression and at which loci methylation differences are a marker of environmental exposures associated with aggression, or the result of aggressive behavior.

Epigenetic studies of aggressive behavior are relatively recent. Classical biomarker studies of aggression focused on blood or urine-based measures, such as lipids, cytokines, and stress hormones, with most consistent results found for lipids. Lipids play an important role in influencing the physical properties of cell membranes and cell signaling in the brain. While studies in humans have only reported associations of altered brain lipid levels and psychiatric disorders such as major depressive disorder or schizophrenia, it is believed that disturbances in lipid homeostasis could cause brain abnormalities and contribute to the development of psychiatric disorders [26]. Serum or plasma levels of lipids in humans and in non-human primates have been frequently explored as potential biomarkers of different types of aggressive behaviors, including violence, self-harm, and suicidal behavior, with violent criminal offenders and suicidal individuals having lower lipid levels as compared with controls [27, 28]. Low serum cholesterol levels have also been associated with early-onset CD disorder in non-violent criminal offenders diagnosed with APD [29]. Eriksen et al. (2017) observed an association of low high density lipoprotein (HDL) at admission of an acute psychiatric ward with violence in both men and women during hospital stay and for men in the first three months post-discharge [30]. Aggression subtypes have also been studied in relation with lipid levels. Conklin and Stanford (2008) reported a relationship of high cholesterol levels with increased premeditated aggression, but no associations of impulsive aggression and lipid levels [31]. Troisi and D'Argenio (2006) found that self-reported verbal aggression, hostility and anger in adults at risk for aggressive behavior was associated with decreased lipid levels; in contrast self-reported physical aggression was associated with an increase in low density lipoprotein (LDL) cholesterol levels only [32]. Most studies looked at associations

between lipids and aggression, but some studies also support a causal effect of lipids on aggression. In monkeys, lowering of cholesterol levels through dietary intervention leads to behavioral changes, most notably in the number of incidents of contact aggression [33]. In humans, an increase in aggressive behaviors tended to be associated with lower levels of total cholesterol and HDL cholesterol. Especially in participants with low baseline aggression, lowering cholesterol levels by statins decreased aggression in younger men (under age 40) but generally increased aggression in postmenopausal women [34].

An interesting connection exists between the 'older' lipid biomarkers of aggression and the 'newer' epigenetic biomarkers, for which a plausible causal biological hypothesis may be formulated. A recent study identified 26 CpGs for which methylation levels were associated with blood lipid levels, and hypothesized that DNA methylation status of circulating immune cells might reflect long-term exposure to blood lipids [35]. To examine the causal relationship between lipid levels and genome-wide DNA methylation a stepwise Mendelian randomization analysis was performed, which showed that differential methylation was the consequence of variation in blood lipid levels and not vice versa. Triglyceride levels had a causal effect on 5 CpG sites, 1 CpG site was causally influenced by LDL and HDL had a causal effect on 2 CpG sites. The CpG sites were associated with expression levels of genes involved in lipid metabolism (CPT1A, SREBF1, DHCR24 and ABCG1), suggesting that methylation of these loci is involved in feedback control of lipid metabolism. These observations led us to hypothesize that the methylation level of these loci in blood can inform us if aggression is associated with long-term exposure to increased or decreased lipid levels: if this is the case, we expect to find that loci where methylation levels are influenced by lipid levels show differential methylation in aggressive individuals, and such loci might complement classic lipid level measures as a biomarker for lipid-related disturbances in aggression.

The aim of the current study was to investigate previously reported and novel biomarkers of adult aggression. We analyzed (1) biomarkers identified in the review by Hagenbeek et al. (2016) [36], namely HDL, LDL and total cholesterol, cholesterol ratios, C-reactive protein (CRP), interleukin-6 (IL-6), and (2) biomarkers which are related to these previously identified lipid biomarkers, i.e. triglycerides, six glucose metabolism markers and inflammatory markers, to test for associations of these biomarkers

with aggression in a large population-based twin-family sample from the Netherlands Twin Register (NTR; N = 5,588). Secondly, we employed a discordant MZ twin design, for which we selected MZ twins discordant for aggression, to test for mean biomarker differences between the MZ discordant twins. The discordant MZ twin design, or co-twin control design, can be viewed as an extension of a case-control design, as its application allows an insight into the origin (confounding effects versus similar genetic/environmental factors) of observed associations between traits [37, 38]. Next, we addressed the hypothesis that aggressive behavior is associated with methylation levels at loci involved in lipid metabolism by testing whether 21 methylation sites associated with lipid levels as reported by Dekkers et al. (2016) [35] are enriched in the aggression EWAS [25]. In a last step, we compared the methylation status of these 21 sites in the aggression discordant twin pairs.

Materials and methods

Subjects

Twins and their family members registered with NTR [39] were visited at home to collect fasting blood and urine samples. Informed consent was obtained from all participants. The NTR Biobank study was approved by the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Centre, Amsterdam (IRB/institute code NTR 03-180).

We selected individuals who were over 18 years with biomarker and aggression data (N = 5,588, range = 18-84 years, mean age at blood sampling = 41.3 years, SD = 13.7, females = 66.6%). The dataset included 3,041 twins, their parents (1,172), siblings (1,067), spouses (291), and offspring (13). There were 1,814 MZ and 1,222 dizygotic (DZ) twins from 1,992 families. Subjects on lipid-lowering medication (N = 683) or who failed to adhere to the fasting protocol (N = 489) were excluded from analyses of lipid levels. For glucose metabolism traits, subjects on medication impacting the Hypothalamic Pituitary Adrenal (HPA)-axis (N = 70), who took diabetes medication (N = 258), or who failed to adhere to the fasting protocol (N = 489) were excluded. For CRP, Tumor Necrosis Factor alpha (TNF-α), IL-6, soluble form of the IL-6 receptor (sIL-6R) and fibrinogen, subjects on anti-inflammatory medication (N = 385) or medication impacting the HPA-axis (N = 70) were excluded.

Aggression data from NTR surveys closest to blood draw were selected. For the majority of individuals aggression was assessed after blood draw (N = 5,196, range: 1-11 years after blood draw, mean = -3.6 year, SD = 1.96), for another 264 participants aggression was assessed prior to the blood draw (range: 1-8 years before blood draw, mean = 2.1, SD = 0.7). Aggression was assessed in the same year as the blood draw for 128 individuals.

Biomarker assessment

Lipids

HDL cholesterol (mmol/l), total cholesterol (mmol/l) and triglycerides (mmol/l) were determined with the Vitros 250 assays (Johnson & Johnson, Rochester, USA) in heparin plasma. LDL (mmol/l) was estimated by the Friedewald formula [40]. HDL-cholesterol values of 6 or higher were excluded. Triglyceride values had a skewed distribution, therefore the values were log-normal transformed prior to outlier removal (values ± 4 SD from the mean).

Glucose metabolism

Insulin (µIU/ml) and glucose (mmol/l) were determined in plasma using the Immulite 1000 Insulin Method (Diagnostic Product Corporation, Los Angeles, USA) and the Vitros 250 Glucose array (Johnson & Johnson, Rochester, USA). Glycated hemoglobin (HbA1c; in percentages) was determined in EDTA plasma using the Nyocard HbA1c assay (Axis-Shield, Oslo, Norway). Homeostatic model assessments (HOMA) quantified insulin resistance and beta-cell function. The homeostatic model assessment of β-cell functioning (HOMA2-B) and insulin resistance (HOMA2-IR) were calculated from the fasting insulin and fasting glucose according to standard formulae [41]. For individuals with glucose values of 7 or higher glucose, insulin and HOMA measurements were excluded; outliers (values ± 4 SD from the mean) were removed for HbA1c. The insulin and HOMA variables were log-normal transformed.

Inflammatory markers

TNF-α (pg/ml), IL-6 (pg/ml) and sIL-6R (pg/ml) were measured in EDTA plasma by UltraSensitive ELISA (R&D systems, Minneapolis, USA, Quantikine HS HSTA00C). The Immulite 1000

CRP assay (Diagnostic Product Corporation, USA) determined CRP levels (mg/l) in heparin plasma. Fibrinogen levels (g/l) were determined by STA Compact Analyzer (Diagnostica Stago, France) in CTAD plasma. Outlying values (> 15 for CRP, IL-6 and TNF-α, > 6 for fibrinogen and > 100,000 for sIL-6R) were excluded. CRP, TNF-α and IL-6 were log-normal transformed [42].

Anthropometric, health and lifestyle measures

Height (cm), weight (kg), hip and waist circumference (cm) were obtained during the home visit and Body Mass Index (BMI: weight [kg] / [height (cm)]^2) and waist-to-hip ratio (WHR) were calculated. Information on medication use in the past six months and smoking status (1, never smokers; 2, former smokers; and 3, current smokers) was also obtained during the home visit.

Aggression assessment

Aggression was assessed by the ASEBA Adult Self-Report (ASR) 'Aggression Syndrome Scale', which sums 15 aggression items, resulting in a sum score ranging from 0 to 30 [43], in NTR Survey 8 (2009) or Survey 10 (2013). ASR aggression data were available for 4,858 individuals for Survey 8 and 3,053 individuals for Survey 10. The survey closest to the age of blood draw was selected when both questionnaires had been filled out [25].

Statistical analyses

We conducted three types of analyses, as detailed below. Correction for multiple testing was done by Bonferroni correction for the number of independent variables (α = 0.05/N independent variables), separately for the confirmatory analyses of the 7 previously described biomarkers [36], and for the exploratory analyses of 10 additional biomarkers and six anthropometric traits, resulting in two distinct alphas for the confirmatory and exploratory analyses. Matrix Spectral Decomposition [44] was applied (implemented in R [45]), to estimate the number independent variables in the correlation matrix of the dependent variables. A correlation matrix between all biomarkers (after applying all exclusion criteria, but not limiting to individuals with aggression data; N = 6,290) was used as input. For the confirmatory analyses five independent dimensions were seen, resulting in an alpha of 0.01. The second set of biomarkers could be reduced to

eleven independent variables, resulting in an alpha of 0.004 for the exploratory analyses. Whenever we refer to marginally significant results, we refer to p-values of $\leq 0.05 \geq 0.01$ for the confirmatory analyses and $\leq 0.05 \geq 0.004$ for the exploratory analyses.

Association of biomarkers and aggressive behavior

To test the association between aggressive behavior and biomarkers, generalized estimation equation (GEE) models were fitted with the R-package GEE using the Gaussian link function for continuous data and the 'exchangeable' option to correct for the correlation structure due to family resemblance using 100 iterations. Three different models were evaluated: in the first model aggressive behavior, sex, age at blood sampling, smoking status, and BMI were the predictors with the lipids, glucose metabolism, and inflammatory markers as outcome variables. In the second model weight, BMI, WHR, hip and waist circumference were the outcome variables with aggressive behavior, sex, age at blood sampling, and smoking status (never, former, current smoker) as predictors. In model 3 height was the outcome variable with aggressive behavior, sex, year of birth, and smoking status as predictors. In models 2 and 3 we explored a potential relationship of the anthropometric traits with aggressive behavior to determine whether these traits should be added as extra covariates to our biomarker model. All models were repeated using a dichotomized 'case/control' aggression variable based on a T-score cut-off of 65, indicating the subclinical threshold for aggressive behavior [43]. For males and females separate sex-specific T-scores were calculated for 18-35 year olds, 36-59 year olds, and individuals over 60 years of age. All GEE models were applied to data in which individuals with missing data on the covariates were excluded.

Discordant monozygotic twin pair analyses

Discordance in MZ twin pairs was defined based on within-pair differences in aggression scores of ≥ 7 points in the survey closest to the moment of blood draw [25]. For MZ pairs with longitudinal aggression data, the within-pair difference had to be at least ≥ 7 points at one survey moment and ≥ 5 points at the other survey moment. Aggression scores were available for both twins in 620 MZ pairs who took part in NTR Biobank, of which 264 pairs had longitudinal data. Based on the criteria

for discordance 31 MZ pairs were discordant for aggression, where discordance was supported by longitudinal data for 6 pairs. Linear regression was used to adjust the biomarkers for the covariates. Mean differences in the residual biomarker levels were tested in a MZ-discordant design by paired t-tests applied in R.

Enrichment analysis

Tab. 1 provides an overview of the 28 lipid associated CpGs by Dekkers et al. [35]. An enrichment test was performed to examine if CpGs associated with lipids have lower p-values for aggression than expected by chance. To this end test statistics from the EWAS of aggression [25] were regressed on a variable indicating whether a CpG is significantly associated with lipids (1) or not (0). These analyses were performed for all CpGs significantly associated with lipids and present in the aggression EWAS (21/28 CpGs).

Results

Characteristics of the aggression data are described in **Tab. 2**. Adult females had higher aggression scores than males (β = 0.444, SE = 0.084, p = 1.07E-07), aggression declined with age (β = -0.026, SE = 0.003, p = 2.98E-18) and 'never smokers' were less aggressive than 'former' or 'current' smokers (β = -0.278, SE = 0.056, p = 8.90E-07). When performing the analyses on a dichotomized aggression phenotype, the association of aggression with smoking was still significant. No association between aggression and

Table 1. Overview of the 28 cytosine guanine dinucleotide (CpG) sites reported by Dekkers et al. (2016) [35] to be significantly associated with lipid levels.

Lipid	cgid	Chromosome	Position
Triglycerides	cg14476101	1	120,255,993
	cg19693031	1	145,441,553
	cg06690548	4	139,162,809
	cg05575921	5	373,379
	cg14817490	5	392,921
	cg06560379	6	44,231,306
	cg19589396	8	103,937,375
	cg07504977	10	102,131,013
	cg11376147	11	57,261,199
	cg00574958	**11**	**68,607,623**
	cg17058475	**11**	**68,607,738**
	cg12556569	11	116,664,040
	cg15863539	17	17,716,951
	cg20544516	17	17,717,184
	cg11024682	17	17,730,095
	cg08857797	17	40,927,700
	cg03636183	19	17,000,586
	cg02711608	19	47,287,965
	cg27243685	21	43,642,367
	cg01881899	21	43,652,705
	cg06500161	**21**	**43,656,588**
LDL-C	**cg27168858**	**1**	**55,351,660**
	cg00908766	1	109,817,497
	cg05119988	4	166,251,190
HDL-C	cg17901584	1	55,353,707
	cg26313301	19	11,219,616
	cg27243685	**21**	**43,642,367**
	cg06500161	21	43,656,588

LDL: low density lipoprotein; HDL: high density lipoprotein.
In bold are the CpGs causally influenced by lipids.

Table 2. Characteristics of the Netherlands Twin Register (NTR) aggression data for Biobank project participants.

Data/analysis group		ASR aggression score						Age at Survey			Age at blood draw			Sex
		N	Mean	Median	SD	Min	Max	Mean	Median	SD	Mean	Median	SD	Female (%)
Survey 8		4,858	2.6	2	2.9	0	24	42	42	14	40	40	14	66.3%
Survey 10		3,053	2.6	2	2.9	0	21	43.6	42	10.9	36.5	35	10.6	69.3
EFA/GEE models[a]		5,588	2.6	2	3	0	24	44.6	42	13.9	41.3	38	13.7	66.6%
Discordant MZ twins	High aggression	31	11.5	10	3.7	7	23	36.7	37	12.4	33.8	33.1	11.7	81%
	Low aggression	31	1.8	1	2.2	0	10	36.8	37	12.3	33.6	33.1	11.5	81%
Extremely discordant MZ twins	High aggression	12	14.5	13	3.9	10	23	32.7	32.5	11.2	29.4	28.8	10.1	66.6%
	Low aggression	12	2	1	2.8	0	10	32.8	32.2	11.1	29.6	29.2	10	66.6%

ASR: Adult Self-Report; EFA: exploratory factor analyses; GEE: generalized estimation equation models; MZ: monozygotic.
[a]All NTR biobank individuals with Survey 8 or 10 data; aggression score and age at survey from the survey closest to date of blood draw.

the anthropometric traits was observed for either the continuous or the dichotomized aggression trait.

Association analyses

For the confirmatory analyses of the 7 biomarkers previously identified in literature and for the 10 related biomarkers in the exploratory analyses, we tested the association with aggression while correcting for sex, age at blood draw, smoking status, and BMI. We observed no association between aggression and biomarkers with either the continuous or dichotomized aggression (**Tab. 3**). To assess if specific items of the aggression scale associated with biomarkers we used exploratory factor analyses (EFA) models in which we included all ASEBA ASR aggression scale items and all blood biomarkers. If blood biomarkers would associate with specific aggression items these would be included in the same factor. However, including the blood biomarkers in the aggression EFA's did not result in interpretable factors, with blood biomarkers clustering in different factors from the aggression items. In the four-factor solutions all aggression items clustered within the same factor and three factors with biomarkers, each including 4 to 8 biomarkers.

Discordant monozygotic twin pair analyses

Mean differences in the residual biomarker levels were tested in 31 MZ discordant twin pairs by paired t-tests. The 'high'-aggressive MZ twin scored on average 9.7 points higher than his or her 'low'-aggressive co-twin (**Fig. 1A**). After correction for multiple testing, we observed a significant mean difference in the exploratory analyses for glucose, with a mean difference of -0.271 (p = 0.003), and a marginally significant mean difference for fibrinogen (mean difference = 0.430, p = 0.01; **Tab. 4**). We checked whether the mean differences in MZ aggression discordant twins could be explained solely by extremely discordant pairs (≥ 10 points difference for aggression score). Twelve MZ pairs met the criteria for being extremely discordant (**Tab. 2**), with on average a 12.5 point difference in aggression scores for the low- versus the high-scoring twin (**Fig. 1B**). The observed trend towards significance for fibrinogen seemed to be driven by extremely discordant MZ twin pairs. After multiple testing, fibrinogen was not significant in the extremely discordant MZ twin pairs, however, we did observe a larger mean

Table 3. Generalized estimation equation (GEE) model results for the association of biomarker levels with aggressive behavior (and covariates).

	N	Estimates					Robust SE					P-value[a]				
		AGG	Sex	Age	Smok.	BMI	AGG	Sex	Age	Smok.	BMI	AGG	Sex	Age	Smok.	BMI
HbA1c	4,988	.0025	.0287	.0065	-.0159	.0078	.0023	.0149	.0005	.0095	.0019	.2833	.0542	2.14E-32	.0951	3.70E-05
Glucose	5,035	-.0021	-.1618	.0106	-.0358	.0287	.0023	.0152	.0006	.0091	.0020	.3612	1.81E-26	4.49E-75	7.84E-05	4.26E-46
Insulin	4,935	-.0031	-.0319	-.0012	-.0244	.0639	.0025	.0168	.0006	.0111	.0022	.2175	.0580	.0469	.0279	1.60E-184
T-cholesterol	4,958	-.0015	.0376	.0322	-.0693	.0251	.0045	.0302	.0011	.0176	.0037	.7375	.2129	8.67E-196	8.67E-05	1.31E-11
LDL	4,953	-.0024	-.0980	.0262	-.0314	.0274	.0040	.0279	.0010	.0165	.0035	.5441	.0004	6.38E-154	.0569	4.08E-15
HDL	4,958	.0012	.2668	.0039	.0160	-.0277	.0016	.0102	.0004	.0065	.0013	.4397	8.38E-151	3.29E-21	.0131	7.88E-106
HDL:CHOL	4,957	.0005	.0500	-.0011	.0062	-.0069	.0004	.0024	.0001	.0015	.0003	.1754	2.87E-93	4.09E-38	3.44E-05	3.33E-117
LDL:HDL	4,952	-.0037	-.5277	.0133	-.0778	.0660	.0041	.0305	.0010	.0179	.0036	.3628	4.86E-67	1.73E-32	1.39E-05	9.18E-71
Triglycerides	4,951	.0000	-.1799	.0035	-.0732	.0360	.0021	.0144	.0005	.0084	.0017	.9883	5.08E-36	1.59E-12	3.09E-18	6.12E-102
CRP	5,100	-.0084	.4177	-.0029	-.1040	.1099	.0053	.0322	.0012	.0198	.0038	.1135	1.89E-38	.0146	1.56E-07	5.34E-183
Fibrinogen	4,973	.0023	.2030	.0075	-.0343	.0462	.0031	.0189	.0008	.0125	.0026	.4739	1.09E-27	1.09E-22	.0060	1.74E-73
TNF-α	5,047	.0021	.092	.0030	-.0026	.0137	.0028	.0178	.0007	.0112	.0023	.4425	7.05E-27	6.12E-06	.8182	2.55E-09
IL-6	5,040	-.0016	.0596	.0098	-.1003	.0416	.0032	.0193	.0007	.0122	.0022	.6195	.0020	8.75E-42	1.58E-16	1.21E-80
sIL-6R	5,064	-36.1944	-1001.0	108.28	27.0819	224.71	52.418	343.86	12.74	208.28	44.15	.4899	.0036	1.95E-17	.1947	3.59E-07
HOMA2-B	4,570	-.0010	.0330	-.0045	.0008	.0262	.0014	.0101	.0004	.0062	.0013	.4895	.0011	9.83E-36	.9026	8.99E-88
HOMA2-S	4,570	.0028	.0502	.002	.0167	-.0519	.0022	.0147	.0005	.0091	.0020	.1933	.0006	.6652	.0671	4.35E-154
HOMA2-IR	4,570	-.0026	-.0511	-.0003	-.0162	.0519	.0022	.0148	.0005	.0092	.0020	.2279	.0006	.6040	.0766	1.12E-154

Smok.: smoking; BMI: Body Mass Index; HbA1c: glycated hemoglobin; LDL: low density lipoprotein; HDL: high density lipoprotein; CRP: C-reactive protein; TNF-α: Tumor Necrosis Factor alpha; IL-6: interleukin-6; sIL-6R: soluble form of the IL-6 receptor; HOMA2-B: the homeostatic model assessment of β-cell functioning; HOMA2-S: the homeostatic model assessment of insulin sensitivity; HOMA2-IR: the homeostatic model assessment of insulin resistance.
[a]p-value threshold corrected for multiple testing for the confirmatory analyses (includes: CRP, LDL, IL6, LDL:HDL ratio and HDL:CHOL ratio and HDL) is p ≤ .01 and p ≤ .004 for the exploratory analyses (anthropometric traits, fibrinogen, insulin, glucose, HOMA2-S, HOMA2-IR, HOMA2-B, HbA1c, sIL-6R, TNF-α and triglycerides).

difference and smaller p-value than for the overall group of discordant MZ pairs (mean difference = 0.669, p = 0.008). The opposite is true for glucose, after excluding the least discordant twin pairs for aggression (Δ ≥ 7 < 10), we no longer observed a significant hit (mean difference = -0.156, p = 0.189). The analysis on extremely discordant MZ pairs also resulted in marginally statistically significant findings for the association between aggression and CRP (mean difference = 1.082, p = 0.016), LDL cholesterol (mean difference = -0.446, p = 0.038), and IL-6 (mean difference = 0.785, p = 0.045). Estimates and p-values for the

analyses including the extremely discordant MZ pairs can be found in **Tab. 4**.

Enrichment analysis of lipid methylation markers in the aggression epigenome-wide association study

None of the CpGs associated with lipids was strongly or significantly associated with aggression in the entire NTR cohort and none of the CpGs showed a significant methylation difference between MZ twins discordant for aggression. CpGs associated with lipids were also not significantly enriched in the EWAS of aggression
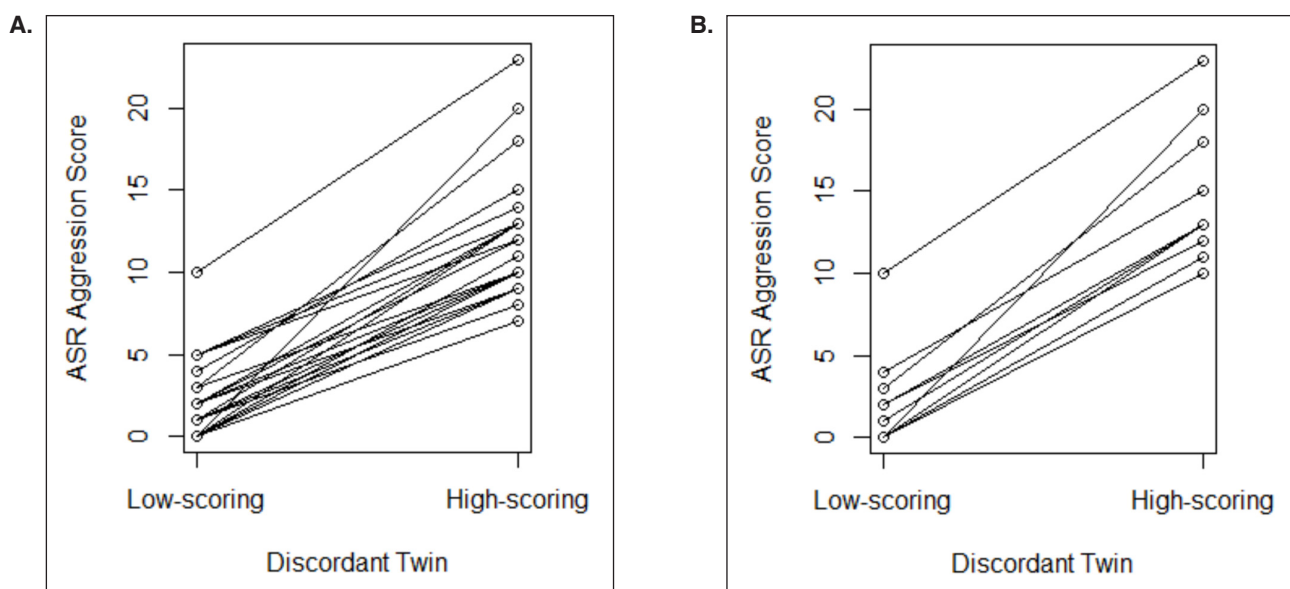

Figure 1. Adult Self-Report (ASR) aggression scores of aggression-discordant monozygotic (MZ) twins. **A.** Aggression scores of discordant MZ twins (≥ 7 points difference). **B.** Aggression scores of extremely discordant MZ twins (≥ 10 points difference).

Aggression discordance is defined as ≥ 7 points difference on the ASR aggression sum score; with extreme discordance defined as ≥ 10 points difference. The ASR aggression scores for the survey closest to blood draw are plotted for low- and high-scoring twins of 31 discordant MZ pairs (**A**) and 12 extremely discordant MZ twin pairs (**B**). The scores of co-twins are connected by lines.

Table 4. Top paired t-test results of residual biomarker levels between aggression discordant monozygotic (MZ) twins.

Biomarker	Low-scoring twin		High-scoring twin		Mean difference[a]	p-value[b]
	Mean	SD	Mean	SD		
Aggression discordant MZ twins (N = 31)						
Glucose	5.3	0.5	5.1	0.5	-0.271	0.003
Fibrinogen	2.7	0.7	3.0	0.8	0.430	0.010
Extremely aggression discordant MZ twins (N = 12)						
Fibrinogen	2.6	0.8	3.0	0.8	0.669	0.008
CRP	2.4	3.1	3.2	3.7	1.082	0.016
LDL	3.2	1.0	2.6	0.8	-0.446	0.038
IL-6	1.5	1.0	2.4	1.7	0.785	0.045

MZ: monozygotic; CRP: C-reactive protein; LDL: low density lipoprotein; IL-6: interleukin-6.
[a]Mean difference in residual biomarker level between aggression-discordant twins; [b]significant after correcting for multiple testing; p ≤ 0.01 confirmatory analyses; p ≤ 0.004 exploratory analyses.

Table 5. Enrichment results for all 21 cytosine guanine dinucleotides (CpGs) associated with lipids in the aggression epigenome-wide association study (EWAS) for the entire Netherlands Twin Register (NTR) cohort and in aggression discordant monozygotic (MZ) twins.

	Estimate	Std. Error	t-value	Pr (> ltl)	bootstrap Std. Error	bootstrap t-value	bootstrap p-value
EWAS of Aggression in the Entire NTR Cohort							
(Intercept)	1.047	0.002	453.841	< 2e-16	0.0023	458.773	< 2e-16
Lipid CpGs	0.385	0.323	1.193	0.233	0.349	1.104	0.269
EWAS in discordant MZ twins							
(Intercept)	0.828	0.0013	825.228	< 2e-16	0.0983	829.161	< 2e-16
Lipid CpGs	0.098	0.1403	0.701	0.483	0.1293	0.762	0.446

EWAS: epigenome-wide association study; NTR: the Netherlands Twin Register; CpGs: cytosine guanine dinucleotides.

in the entire NTR cohort or in discordant MZ twins (**Tab. 5**).

Discussion

We examined the association of lipid levels with adult aggression in a large population-based sample and in discordant MZ twin pairs. No significant associations were seen, though the analysis in the MZ twins extremely discordant for aggression found a marginally significant (p = 0.038) association of decreased LDL cholesterol levels in the more aggressive twin, congruent with previous studies showing decreased LDL levels for aggression [46] and suicidality in younger individuals [28]. However, physical aggression was previously characterized by increased LDL levels [32]. In 31 pairs of MZ twin pairs discordant for aggression we identified a significant association with glucose levels, where the twins with high aggression had decreased glucose levels. The analyses in MZ twins extremely discordant for aggression also revealed marginally significant results for cytokine levels, where elevated cytokine levels were observed for the high-scoring twins. Dekkers et al. [35] used Mendelian randomization to show that circulating lipid levels influence DNA methylation and a study of methylation and adult aggression found suggestive evidence for several associations [25]. We hypothesized that the methylation level of lipid loci in blood can inform us if aggression is associated with long-term exposure to increased or decreased lipid levels, but found no significant enrichment of the lipid CpG sites in the aggression EWAS [25].

Discordant MZ analyses identified low glucose as potential biomarker for aggression. Glucose levels in high-scoring twins ranged from 4.4-6.4, with mean glucose levels of 5.1 (SD = 0.5), while

these levels ranged from 4.3-6.5 in low-scoring twins with a mean of 5.3 (SD = 0.5). The observed low glucose levels in the more aggressive MZ twins is no longer significant when concentrating on the 12 MZ twin pairs extremely discordant for aggression. One explanation might be reduced power, an alternative explanation might be an increase of insulin resistance in these twins, leading to higher, rather than lower, glucose levels. However, while no longer significant, we still observe a trend for lower glucose levels in the more aggressive MZ twins. In addition, insulin resistance itself (HOMA2-IR) is not significantly associated with aggression in either of GEE analyses (**Tab. 3**) or our discordant MZ analyses. Similar to the current study, in a sample of 107 married couples, small but significant associations of low evening glucose levels with both aggressive impulses (β = -0.008, t(213) = -2.82, p = 0.006) and aggressive behavior (β = -0.003, t(206) = -5.63, p < 0.0001) were observed [47]. The low glucose levels as observed in the current study seem in contrast with previous studies reporting increased aggression in individuals with diabetes [48] or metabolic syndrome [49], both of which are characterized by high glucose levels. Together this might indicate that the relationship of glucose and aggression is non-linear, with aggressive behavior observed on both ends of the glucose distribution. We excluded individuals on diabetes medication (N = 258) from the analyses. For individuals on diabetes medication, aggression scores on Survey 8 were available for 92 individuals with a range of 0-8 and a mean aggression score of 1.9 (SD = 2.4). Survey 10 data were available for 23 individuals with aggression scores ranging from 0 to 6 (mean = 2.2, SD = 1.8). Aggression scores in these individuals were on average similar to the aggression scores observed in the total sample although the range is restricted in comparison.

Within-pair differences for fibrinogen levels were marginally significant and showed a higher mean difference of 0.669 in extremely discordant pairs as compared to 0.430 in the total group of 31 discordant pairs. Fibrinogen levels range from 1.91-4.80 in the high scoring twins (mean = 3.0, SD = 0.8) and 1.62-4.12 in the low-scoring twins (mean = 2.7, SD = 0.7); similarly, in the 12 extremely discordant pairs these ranges were 2.23-4.30 (mean = 3, SD = 0.8) and 1.62-4.00 (mean = 2.6, SD = 0.8), respectively. In the 12 extremely discordant MZ twin pairs we also observed a trend for increased CRP and IL-6 levels in the high-scoring twins. High-scoring twins had mean CRP values of 3.2 (SD = 3.7) and mean IL-6 values of 2.4 (SD = 1.7) as compared with low-scoring twin with mean CRP values of 2.4 (SD = 3.1) and mean IL-6 values of 1.5 (SD = 1). Both human and animal studies have identified elevated cytokine levels in aggressive behavior [50]. This association was also observed for psychiatric disorders closely related to aggression, such as intermittent explosive disorder (IED) [51], personality disorders in adults [52] or severe affective and behavioral dysregulation in children [53]. However, lower levels of both pro- and anti-inflammatory cytokines also were observed in young adult males with a childhood chronic physical aggression trajectory [54]. Despite this contradiction it would appear that cytokine levels are associated with aggression and associations also were observed on an epigenetic level. Provençal et al. (2013) reported differential methylation of cytokine genomic loci and transcription factors in young adult males with a history of chronic physical aggression as compared with controls [54]. These loci were not replicated in a later EWAS study [25] which used a different technique to measure DNA methylation. While we found no evidence that the association of methylation and adult aggression is due to lipid levels, it is possible that variation in cytokine levels might explain this association.

This study has several strengths and limitations. It is one of the most comprehensive investigations of the role of classic biomarkers in aggression, utilizing a population-based sample, the powerful MZ discordant design, and including 17 blood biomarkers. While our large study represents biomarkers from three different classes, this list is far from exhaustive. Extensive research has been done to elucidate the role of neurotransmitters and hormones in aggression; with several hypotheses stating that the imbalance between hormones and neurotransmitter levels is responsible for aggressive behavior. For example, Montoya et al. (2012) proposed a dual-hormone serotonergic hypothesis [55], in which a high ratio of testosterone to cortisol levels is responsible for a general predisposition towards aggression with low prefrontal serotonergic functioning specifically predisposing towards social aggression. We might thus miss vital pieces of information in limiting our study to three classes of blood biomarkers. Future studies should attempt to be even more encompassing in the inclusion of biomarkers; preferably by including high-throughput techniques such as metabolomics. Such studies should consider using multiple metabolomics platforms in their design in order to obtain a detailed account of various biochemical classes and their role in aggression. In addition to expanding the number of biochemical classes, we also recommend to study the biochemistry of aggression in other, preferably multiple, tissues, as metabolic flux is predicted to be tissue specific [56].

A limitation of a population-based sample is the lack of extremely aggressive individuals. While our sample covers both the normal and clinical range of the ASEBA ASR aggression scale [43], few individuals have (sub-)clinical aggression scores (T-scores > 65). We addressed this limitation by inclusion of the MZ discordant design. However, even in a large twin population, there were relatively small numbers of discordant twin pairs, as aggression is a heritable trait. Another strength of the current study is that it included subjects of a wide age-range (18-84 years) encompassing the human lifespan of early to late adulthood. However, this is a cross-sectional dataset which does not cover the entire human life-span. Currently, we can make no inferences about the role of biomarkers in childhood or adolescent aggression; where the biological underpinnings of aggression might differ from adult aggression. For example, for epigenetics, sex- and age-specific variation in the influence of genetic and environmental factors on methylation has been observed [57]. A next step will be to examine the role of biomarkers and epigenetics in aggression across the lifespan, including childhood and adolescence.

A third strength is the comprehensive range of statistical methods used to elucidate the role of biomarkers in aggression, including EFA to investigate whether including biomarkers and aggression scale items in the same EFA would result in biologically interpretable factors. However, in the resulting four-factor model, biomarkers and aggression items clustered in separate factors.

Furthermore, the EFA with the best fit for the models including only the aggression items resulted in a three-factor solution. This is in contrast with the factor structure of childhood aggression, for which two aggression subtypes, relational and direct aggression, were identified [58]. A recent study suggested to discontinue the use of subtypes in disruptive behavior problems such as aggression and identified in factor mixture models a one-class solution, suggesting we should move from distinct subtypes to a multidimensional approach to disruptive behavior problems [59]. Incorporating a multidimensional approach to biomarkers studies could lead towards improved diagnostic capabilities and aid in the development of 'personalized medicine' approaches for the treatment of aggression.

In conclusion, in MZ twin pairs discordant for aggression we found a significant association for lower glucose levels and a trend for higher fibrinogen levels in the twins scoring high for aggression. Repeating the analyses in 12 pairs of extremely aggression discordant MZ twin pairs also resulted in marginally significant findings for CRP, IL-6 and LDL cholesterol, while the association for glucose disappeared completely. We found no evidence for enrichment of lipid CpG sites in the aggression EWAS in the entire NTR cohort of in MZ twins discordant for aggression nor were lipids significantly associated to adult aggression in a large population-based sample.

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Declaration of interest

The Authors declare no competing financial interests.

Supplementary Material

Supplementary Material of the paper can be found on NTR website at the following link: http://www.tweelingenregister.org/publicaties/wetenschappelijke-publicaties/supplementary-materials/.

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