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Full-length Article

Exploring the use of immunomethylomics in the characterization of depressed patients: A proof-of-concept study

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ABSTRACT

Alterations in DNA methylation and inflammation could represent valid biomarkers for the stratification of patients with major depressive disorder (MDD). This study explored the use of DNA-methylation based immunological cell-type profiles in the context of MDD and symptom severity over time.

In 119 individuals with MDD, DNA-methylation was assessed on whole blood using the Illumina Infinium MethylationEPIC 850 k BeadChip. Quality control and data processing, as well as cell type estimation was conducted using the RnBeads package. The cell type composition was estimated using epigenome-wide DNA methylation signatures, applying the Houseman method, considering six cell types (neutrophils, natural killer cells (NK), B cells, CD4+ T cells, CD8+ T cells and monocytes). Two cytokines (IL-6 and IL-1β) and hsCRP were quantified in serum. We performed a hierarchical cluster analysis on the six estimated cell-types and tested the differences between these clusters in relation to the two cytokines and hsCRP, depression severity at baseline, and after 6 weeks of treatment (celecoxib/placebo + vortioxetine). We performed a second cluster analysis with cell-types and cytokines combined. ANCOVA was used to test for differences across clusters. We applied the Bonferroni correction.

After quality control, we included 113 participants. Two clusters were identified, cluster 1 was high in CD4+ cells and NK, cluster 2 was high in CD8+ T-cells and B-cells, with similar fractions of neutrophils and monocytes. The clusters were not associated with either of the two cytokines and hsCRP, or depression severity at baseline, but cluster 1 showed higher depression severity after 6 weeks, corrected for baseline ($p = 0.0060$). The second cluster analysis found similar results: cluster 1 was low in CD8+ T-cells, B-cells, and IL-1β. Cluster 2 was low in CD4+ cells and natural killer cells. Neutrophils, monocytes, IL-6 and hsCRP were not different between the clusters. Participants in cluster 1 showed higher depression severity at baseline than cluster 2 ($p = 0.034$), but no difference in depression severity after 6 weeks.

DNA-methylation based cell-type profiles may be valuable in the immunological characterization and stratification of patients with MDD. Future models should consider the inclusion of more cell-types and cytokines for better a prediction of treatment outcomes.

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1. Introduction

The presence of an inflammatory subtype of depression, also referred to as 'immunometabolic' depression, has been repeatedly suggested (Drevets et al., 2022; Milaneschi et al., 2020). Multiple studies in different fields provided independent evidence on the involvement of inflammation in major depressive disorder (MDD) (Milaneschi et al., 2020) and treatment effects (Benedetti et al., 2022; Maffioletti et al., 2020). In the context of personalised medicine, researchers and clinicians are highly interested in biomarkers able to stratify patients according to their inflammatory subtype, which could have important implications on treatment, as several immunomodulatory agents have been explored as augmentation strategies in MDD (Drevets et al., 2022; Fourrier et al., 2018). However, there is still no clear strategy on the best approach to identify and stratify depressed individuals based on the degree and type of inflammatory component (Drevets et al., 2022; Milaneschi et al., 2020). This is challenging given the high heterogeneity of MDD, and the available studies did not provide clinical applications yet (Drevets et al., 2022).

Immunological markers in blood (Himmerich et al., 2019; Felger et al., 2020) and cerebrospinal fluid (CSF) (Felger et al., 2020; Enache et al., 2019) have been suggested as possible biomarkers for the study of the inflammatory component of MDD. Interleukin 6 (IL-6), which shows both pro- and anti-inflammatory capacities (Scheller et al., 2011), seems to play a consistent and central role in the link between inflammation and depression (Howren et al., 2009). However, cytokines do not act alone but interact amongst each other, and others were particularly studied in the context of MDD, namely Interleukin 1-beta (IL-1β), and Tumor Necrosis Factor-alfa (TNF-α), as well as C-reactive Protein (CRP) (Himmerich et al., 2019; Felger et al., 2020; Howren et al., 2009). Despite some negative findings (Lamers et al., 2019), interleukin-6 (IL-6) or/and CRP were associated with relevant phenotypes of depression, such as depression severity and risk of chronicity, and the risk of developing depressive symptoms in longitudinal studies (Mac Giollabhui et al., 2021). When considering the role of these biomarkers in depression, concomitant factors involved in the modulation of systemic inflammation should be taken in account, such as body mass index (BMI) (Suneson et al., 2023; Milaneschi et al., 2019). However, the relationship between inflammation and depression seems to be robust, despite the potential mediating role of BMI (Howren et al., 2009; Lamers et al., 2019; Ambrósio et al., 2018).

Immune cell-types and cell-type composition have been linked to depression: a *meta*-analysis showed that an increased mean absolute count of white blood cells was associated with depression, as well as a number of specific cell-types, including granulocytes, neutrophils, monocytes, CD4+ T-cells, natural killer cells, B cells, and activated T-cells (Foley et al., 2023). The CD4+/CD8+ T cell ratio and monocyte count were linked to the severity of MDD as well (Zhou et al., 2022). An inflammatory MDD subtype was described as having an increased number of neutrophils, monocytes, and CD4+ T-cells, in combination with increased CRP and IL-6 (Lynall et al., 2020).

Other biomarkers, such as mRNA (Cattaneo et al., 2020; Zang et al., 2023) and DNA methylation were suggested to pick-up this inflammatory signal in depressed patients as well (Crawford et al., 2018; Chan et al., 2020), and they were also linked to treatment outcome (Cattaneo et al., 2020; Zang et al., 2023). A network co-methylation analysis with a self-reported history of depression as outcome found a module enriched for immune-related pathways. This module implied a primary role of IL-6 in the association between inflammation and depression (Crawford et al., 2018).

As DNA-methylation is cell-type specific, cell-type composition is highly relevant in studies examining this biomarker. Cell-type composition can be estimated using models based on epigenome-wide DNA methylation (Houseman et al., 2014; Salas et al., 2018), providing exciting opportunities in the so-called field of 'immunomethylomics', where the focus is primarily on the estimated immunological cell-types in whole blood (Salas et al., 2018; Titus et al., 2017). Little is known about the possible use and implications of these approaches in the context of depression, as potential biomarkers of disease manifestations and treatment response. Despite the limitations of methylation-based cell-type estimates (uncertainty in the estimates, collinearity among the relative compositions (Teschendorff and Zheng, 2017; Qi and Teschendorff, 2022), immunomethylomics represents an accessible way for immunological profiling and exploration of inflammation states in the context of depression.))

The study of cell-type profiles estimated from epigenome-wide DNA methylation has been poorly explored in the context of depression and inflammation. One study linked the estimated cell-types to accelerated DNA-methylation age (DNAmAge) and found a reduction in natural killer cells in untreated patients with depression (Shindo et al., 2023). In other fields of medicine with a more extensive tradition in histopathology, e.g., cancer research, this approach has shown merit already (Chen et al., 2022; Shanthikumar et al., 2021; Lee et al., 2024). As estimated cell-type proportions potentially harbour a lot of information in their combined interpretation, this study aimed to explore the link between estimated cell-type proportions and depression in the context of inflammation through hierarchical clustering of the cell-types. We considered the estimation of six cell-types: neutrophils, natural killer cells, B cells, CD4+ T cells, CD8+ T cells, and monocytes (Salas et al., 2018).

In line with the current literature, we hypothesised cell-type clusters to be associated with two of the cytokines most consistently associated with inflammation and depression, namely IL-6 and IL-1β, and high-sensitive CRP (hsCRP). We also tested if these cell-type profiles are associated with depression severity at baseline, and with depression severity over time, as immunological cell-types are linked to inflammation, which is a process spanning over time. Finally, we performed hierarchical clustering combining the estimated cell-types, the two cytokines, and hsCRP, expecting that it will perform better than clustering based on the estimated cell-type proportions alone. To our knowledge, this is the first study to analyse DNA-methylation based cell-type profiles and their combination with cytokine levels in the context of depression severity.

2. Materials and methods

2.1. Sample description

At baseline, our sample consisted of 119 individuals with Major Depressive Disorder (MDD), according to DSM-IV-TR (American Psychiatric Association, 1998). Data were collected in Adelaide, Australia between 2017 and 2020 as part of a longitudinal cohort for a randomised trial (Fourrier et al., 2018). As part of this trial, all individuals were treated with the antidepressant Vortioxetine. Included patients were randomised for an augmentation treatment with Celecoxib (10 mg) or placebo. After quality control (QC), as described below, 113 individuals were included in the analyses.

After quality control (QC), as described below, 113 individuals were included in the analyses. Though recruitment was focused on inflammation state (hsCRP>3.0 mg/L vs. hsCRP<3.0 mg/L) this information was not included in our first analysis as the focus was on immunomethylomics. We included hsCRP and both cytokines as variables in the second cluster analysis to account for the recruitment strategy (hsCRP) and extend the analysis with both cytokines of interest (IL-6, IL-1β). Only baseline data were used for the molecular markers. The sample had a mean age of 44 years (range 18–75) and consisted of 56 % of women (N=63). Depression severity was measured using the Montgomery-Åsberg Depression Rating Scale (MADRS) at baseline and after the treatment period with celecoxib, i.e., 6 weeks. The mean MADRS at baseline was 27.7 (SD=6.5), reflecting moderate to severe depression for most participants. In the context of the cohort, longitudinal phenotypic data are available as well, including MADRS after a 6-week interval,

following treatment (M=20.2; SD=10.8). For a detailed description of the sample and trial, we refer to Fourrier et al. (Fourrier et al., 2018). Relevant exclusion criteria include the presence of a co-morbid psychiatric or addiction disorder, as well as primary inflammatory or immune-related disorders. Furthermore, patients with a neurodegenerative disorder or a history of a neurological disorder were excluded, as well as patients with relative contra-indications for Vortioxetine or Celecoxib, such as a history of gastro-intestinal bleeding. Any other disorder that did not lead to exclusion of the patient was registered and considered as 'medical comorbidity'. These include any known medical condition reported by the patient, such as orthopedic injuries and their treatment, hypercholesterolemia, endocrinological conditions, asthma and COPD, benign tumors, etc.

The study and data collection has been approved by the human research ethics committees of the Royal Adelaide Hospital and the University of Adelaide (reference number R20170320 HREC/17/RAH/111), and pre-registered on the Australian New Zealand Clinical Trials Registry (ACTRN12617000527369; https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=12617000527369p). In addition, patients provided written consent prior to commencing any study procedures.

2.2. Assessment of immunomethylomics, hsCRP, and cytokines

All 119 participants provided whole blood for DNA-methylation analysis with the the Illumina Infinium MethylationEPIC 850 k BeadChip. DNA was isolated from whole blood samples using standard procedures (QIAamp DNA Blood Midi-Kit, Qiagen, Hilden, Germany) followed by purification (Amicon 0,5ml 3 K; Merck/Millipore, Darmstadt, Germany) and pipetting on 96-well plates for chip-based analyses. Bisulfite conversion and handling of the DNA methylation chips were performed in the Life&Brain Institute Bonn (Zillich et al., 2022). Samples were randomized on plates and chips based on patients' sex, age, and treatment. Following analysis on HiScan array scanning systems (Illumina, San Diego, CA), data were transferred as.idat files.

As genomic variants are available from the described participants, they were used to estimate principal components that reflect ancestry (see supplementary figure S1) to include in the subsequent analyses.

The further processing and QC measures of the epigenome-wide DNA methylation data was performed using R (version 4.3.1) and the 'RnBeads' pipeline (Package RnBeads 2.0, (Assenov et al., 2014; RnBeads 2.0: comprehensive analysis of DNA methylation data | Genome Biology | Full Text [Internet], 2023). Following QC procedures, 113 participants were included for the baseline measurement (for details on QC, see supplementary materials, Table S1). Using the available epigenome-wide DNA-methylation data, immunological cell-types were estimated using the Houseman method (Houseman et al., 2014) through the build-in RnBeads command (rnb.execute.ct.estimation(), settings: test.max.markers = 10000, top.markers = 500). The dataset GSE110554 as published by Salas et al. (Salas et al., 2018) as a validated reference was used for the estimation of the proportions of the 6 cell-types of interest (neutrophils, natural killer cells (NK), B cells, CD4+ T cells, CD8+ T cells, monocytes). An estimate of a proportion for each of the cell-types was returned for each of the individuals, based on the distinct methylomic signature for each cell-type. The raw coefficients were used for the analysis.

The cytokines of interest for this study (IL-1β and 1L-6) were obtained using serum. IL-1β and 1L-6 were analysed with the LEGENDplex™ Human Inflammation Panel 1 (BioLegend in line with the manufacturer's manual. Raw data were analysed using the Qognit software (BioLegend®; version 2022–07-15). Further QC-steps include a minimum of 50 for the bead-count and a maximum of 30 for the coefficient of variance (CV). All samples of the individuals were in triplo on the assays, with all samples of one individual on the same assay. The mean value of at least two out of three samples were used for the final concentration. Furthermore, raw data were standardised using a positive control to reduce batch effects, quantile normalised, log-transformed, and residualised for the statistical analyses. For hsCRP we relied on the BNTM II System (Siemens Healthcare GmbH, Erlangen, Germany) with serum and the reagent "N CardioPhaseTM hsCRP" (#OQIY13/10446090, Siemens Healthcare GmbH, Erlangen, Germany) as used for immunonephelometry at the central laboratory of the University Hospital Münster (UKM).

2.3. Statistical analyses

All cell-types, hsCRP, and cytokines were scaled to perform a hierarchical clustering on 1) the six cell-types combined and 2) the six cell-types, hsCRP, and the two cytokines combined. For the hierarchical clustering, Euclidian distances were estimated, and the 'ward' method was used in form of 'ward.D', as we are not interested in strengthening the effect of longer distances, we decided against methods using squared distances (e.g., squared Euclidean distances or ward.D2). We used to GAP-statistic to decide on two clusters for the best fit for our data (see supplementary data and fig. S2).

Following cluster identification based on the six estimated cell-types, ANCOVA was used to explore their characteristics. Relevant confounding variables included sex, age, BMI, years of education as a proxy for socioeconomic status, and the presence of any other reported medical comorbidity (yes vs. no). Ancestry was estimated by genome-based principal components (PC1 and PC2). As shown in supplementary figure S1, ancestry is very homogeneous in this sample and did not differ between the identified clusters (PC1: $F(1,111) = 0.10$, $p = 0.75$; PC2: $F(1,111) = 0.03$, $p = 0.87$). Therefore, it was not included in the regression analyses.

In light of our hypothesis, the relationship between the clusters, hsCRP, and available cytokines was explored (i.e., IL-1β and IL6), as well as depression severity (MADRS) at baseline and end of RCT (i.e., 6 weeks), controlled for baseline. We counted a total of four hypotheses to test, resulting in a Bonferroni correction for multiple testing of $p < 0.0125$.

Descriptive statistics on demographic variables (Table 1) were performed with Fisher's exact test and ANOVA.

3. Results

3.1. Cluster analysis

The optimal solution for the hierarchical clustering analysis of the six cell-types resulted in two clusters. The characteristics of the clusters are described below in Table 1 and Fig. 1.

Cluster 1 shows a relative underrepresentation of CD8+ T and B-cells, whereas CD4+ T and NK are relatively overrepresented as compared to cluster 2. Therefore, cluster 1 will be called "CD4+/NK" and cluster 2 "CD8+/B".

3.2. Interplay of cell-types and cytokines per cluster

The selected cytokines (IL-6 and IL-1β), and hsCRP did not show a significant association with the identified clusters, nor in a model with all three predictors, nor in models looking at the individual cytokines separately. Fig. 2 shows the clusters in relation to each of the combinations of hsCRP and the two cytokines of interest. All models were corrected for sex, BMI, age, years of education, and other medical comorbidity. Sex-stratified sub-analyses did not show any significant results either. Full models and results are presented in the supplementary data (Table S2).

3.3. Cell-types and depression at baseline

No significant association was found between the cell-type based clusters and depression at baseline (model corrected for all named

Table 1
Description of both clusters. Summary of cluster 1 (high in CD4+ T-cells and natural killer cells (NK)) and cluster 2 (high in CD8+ T-cells and B-cells). Both clusters are well comparable regarding standard demographics, including sex, age, BMI, years of education, and the presence of any medical comorbidity. Cell-type proportions, hsCRP (mg/L), and cytokines (pg/mL) are described as non-scaled, raw coefficients, concentrations, and standardized concentrations, respectively.

	Cluster 1: CD4+/NK	Cluster 2: CD8+/B	
Sample			
N	*N*=43	*N*=70	
% women (*N*)	65 % (28)	N=50 % (35)	$p = 0.12$
Age (*M*; *SD*)	42.5 (14.2)	45.6 (14.2)	$F(1,111) = 1.27$, $p = 0.26$
BMI (*M*; *SD*)	30.2 (7.9)	29.5 (6.7)	$F(1,111) = 0.25$, $p = 0.62$
Years of education (*M*; *SD*)	13.6 (2.6)	14.3 (2.0)	$F(1,111) = 2.85$, $p = 0.09$
Presence of medical Comorbidity (yes/no)	37/6	62/8	$p = 0.77$
hsCRP > 3 mg/L (*N*; %)	16; 37 %	21; 30 %	$p = 0.54$
(*) Intervention group (celecoxib (%))	25 (58 %)	30 (43 %)	$p = 0.13$
MADRS (baseline)	28.7 (7.2)	27.2 (5.9)	$F(1,111) = 0.36$, $p = 0.22$
Estimated cell-type proportions, hsCRP, and cytokines			
B-cells (*M*; *SD*)	0.07 (0.02)	0.08 (0.02)	$F(1,111) = 11.87$, $p < 0.001$
Monocytes (*M*; *SD*)	0.10 (0.02)	0.10 (0.03)	$F(1,111) = 0.95$, $p = 0.33$
Neutrophils (*M*; *SD*)	0.60 (0.10)	0.62 (0.07)	$F(1,111) = 0.71$, $p = 0.40$
Natural Killer-cells (*M*; *SD*)	0.00 (0.03)	−0.04 (0.03)	$F(1,111) = 78.73$, $p < 0.001$
CD4-T (*M*; *SD*)	0.10 (0.06)	−0.06 (0.07)	$F(1,111) = 163.1$, $p < 0.001$
CD8-T (*M*; *SD*)	0.20 (0.06)	0.39 (0.09)	$F(1,111) = 158.1$, $p < 0.001$
hsCRP (median; *SD*)	1.80 (4.97)	1.23 (3.90)	*CI*:[−0.30, 1.10], $p = 0.31$
IL-1β (median; *SD*)	0.0082 (0.16)	0.012 (0.22)	*CI*:[$-3.05\text{x}10^{-5}$, $5.66\text{x}10^{-3}$], $p = 0.83$
IL-6 (median; *SD*)	0.045 (0.19)	0.055 (0.21)	*CI*:[−0.022, 0.019], $p = 0.92$

(*) As we included MADRS after the 6-week intervention, mode of intervention (celecoxib vs. placebo) was described as well in the demographics table. No statistical difference was seen between both clusters as based on the estimated cell-types using baseline DNA-methylation.

confounding variables: $F(1,106) = 1.54$, $p = 0.22$), see also Fig. 3 (A). Sex-stratified analyses did not show any significant association either. Full models are presented in the supplementary data (Table S3, fig. S3).

3.4. Cell-types at baseline and depression severity over time (6 weeks)

The model, controlled for depression severity (MADRS) at baseline, sex, BMI, education and the presence of another medical comorbidity, showed a significant association with depression severity after the intervention at 6 weeks ($F(1,105) = 7.99$, $p = 0.0056$). The CD4+/NK-cluster showed more severe depression after 6 weeks as compared to the CD8+/B cluster. This result remained robust as well after additional correction for treatment modality (celecoxib vs. placebo; $F(1,105) = 8.0$, $p = 0.0056$) and is below our cut-off for multiple testing ($p < 0.0125$). The explained variance (R^2) of the cell-type clusters on depression severity after 6 weeks is estimated to be 0.070 ($F(3,109) = 3.80$, $p = 0.012$; corrected for severity at baseline and sex). Group differences per Cluster, as well as the relationship of estimated cell-type composition and depression severity at week 6, are shown in Fig. 3. A sex-stratified analysis showed that this effect is primarily present in women ($F(1,56) = 6.95$, $p = 0.011$), as compared to men ($F(1,43) = 2.06$, $p = 0.160$).

3.5. Re-clustering with cell-types, hsCRP, and cytokines

For the hierarchical re-clustering with the cell-types enriched with the two cytokines and hsCRP, we stuck to the two-cluster solution, despite it being second best after the single cluster solution (supplementary data Fig. S4). Both clusters showed high overlap with the clusters of the first hierarchical clustering analysis (Fig. 4); 88.5 % of individuals (*N*=100) stayed in their respective clusters. The first cluster was very similar to the cluster CD4+/NK and was named "CD4+/NK-2" as it was also high in CD4+ T-cells and natural killer cells. It consists of 38 individuals with four individuals that moved from cluster CD8+/B to CD4+/NK-2. The second cluster was very similar to cluster CD8+/B, though also relatively high in IL-1β and was therefore called "CD8+/B/IL-1β". This cluster consisted of 75 individuals with 9 individuals having moved in the opposite direction, from CD4+/NK to CD8+/B/IL-1β. Neutrophils, monocytes, IL-6 and hsCRP were not statistically different between the two clusters. Demographics of clusters CD4+/NK-2 and CD8+/B/IL-1β are shown in the supplementary data, Table S5. Both clusters were comparable for sex, age, BMI, and the presence of co-morbidity. CD8+/B/IL-1β showed higher education than cluster CD4+/NK-2 ($F(1,111) = 3.95$, $p = 0.049$).

This cluster solution was associated with depression severity at baseline, though not withstanding correction for multiple testing. This was the case in a model corrected for age, BMI, years of education, sex, and the presence of comorbidity ($F(1,106) = 4.46$, $p = 0.037$). Cluster CD4+/NK-2 showed higher depression severity at baseline. However, clusters were not different for depression severity after 6 weeks, corrected for all confounding variables, including baseline severity ($F(1,105) = 3.33$, $p = 0.071$).

4. Discussion

Our results provide the first evidence that immune-related cell-type profiles based on epigenome-wide DNA-methylation may be a valuable biomarker in depression. A hierarchical clustering model distinguished a CD4+/NK cluster, with a relative underrepresentation of CD8T and B-cells, and CD4T and NK cells overrepresentation as compared to cluster 2, which was named CD8+/B. These clusters showed different depression severity after 6 weeks of treatment, independent of depression severity at baseline or treatment modality (celecoxib vs. placebo). Individuals in the CD4+/NK cluster showed indeed more severe depressive symptoms at week 6, with sex-differences, which is in line with the prior literature, though primarily discussed in relation to IL-6 (Lamers et al., 2019). Contrary to our expectations, however, the identified clusters were not associated with IL-6, IL-1β or hsCRP in our sample. Neither cluster was associated with depression severity at baseline, i.e., the time-point of blood collection for DNA methylation.

In a second cluster analysis we integrated estimated cell-types, hsCRP, and the two cytokines of interest. IL-1β played an additional discriminatory role in this analysis. These clusters were associated with depression severity at baseline, with again higher depression severity in individuals with high fractions of CD4+ T-cells and NK-cells, though not withstanding multiple testing correction. This cluster solution was not associated with depression severity after 6 weeks of treatment. This discrepancy between the results of the two cluster analyses may be due the different nature of the considered biomarkers, i.e., only estimated cell-types in the first cluster analysis and estimated cell-types + cytokine levels in the second one. At this point, pre-existing data that compare cell-type-models with or without cytokines at the same time-point, are still scarce. Hence, every interpretation is highly speculative and creates new hypotheses to be tested. One hypothesis, that needs further testing in a sample with a longer time span, is that cytokines may fluctuate rapidly and reflect inflammation levels in a certain moment (in this

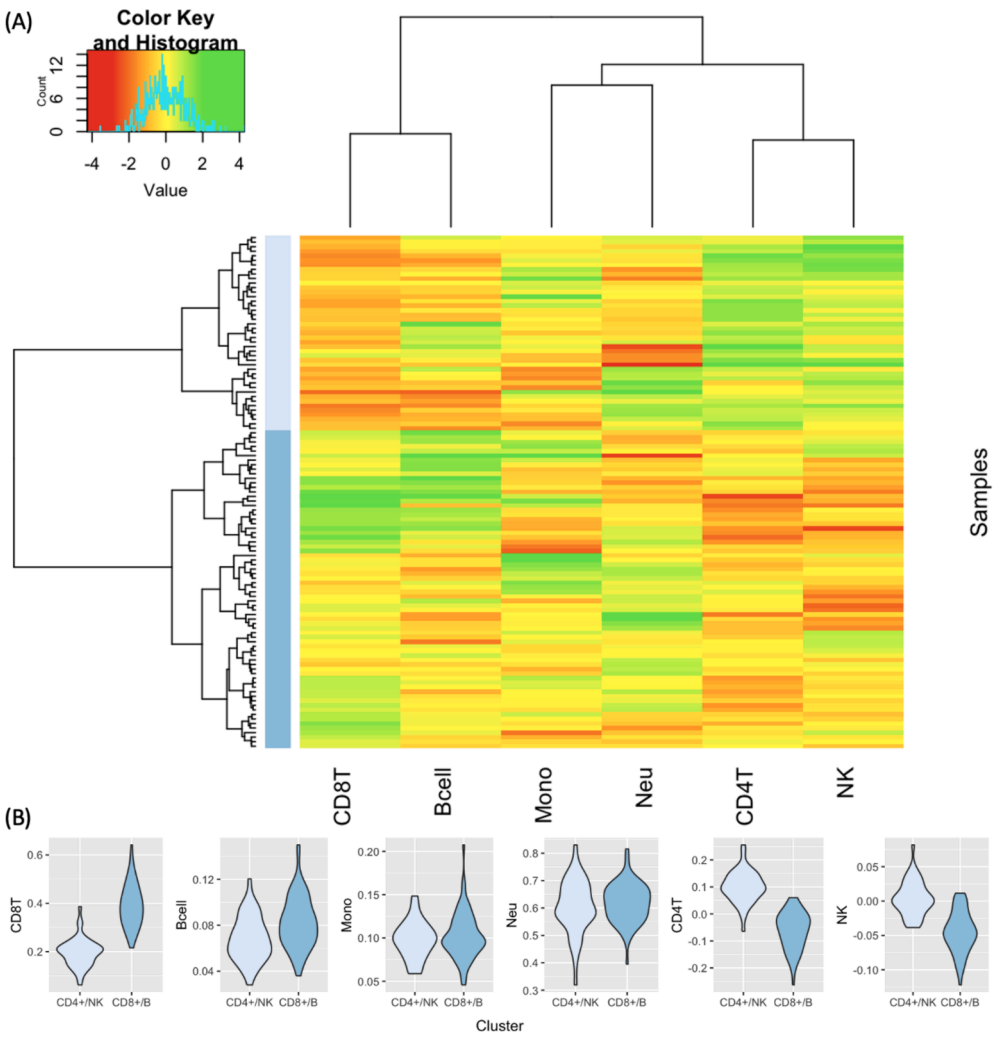

Fig. 1. Result of Hierarchical clustering. (A) Result of hierarchical clustering analysis based on six cell-types reflecting the epigenome-wide signature at baseline. (B) Composition of clusters per estimated cell-type fraction (raw coefficients).

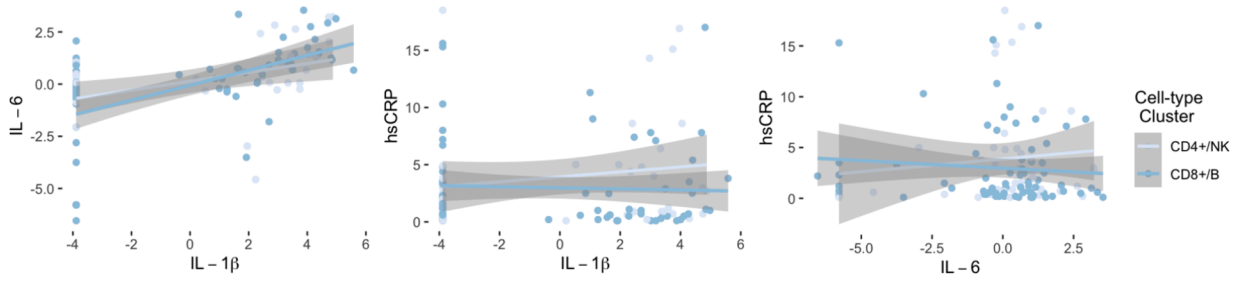

Fig. 2. Cytokines, hsCRP, and clusters. As the two cytokines and hsCRP are known to interact, the clusters are depicted in relation of each combination of two out of three cytokines of interest.

study, baseline), whereas the estimated cell-types may reflect inflammation in the longer term, with impact on depression severity over time.

Previous research linked monocytes and neutrophils to depression, in particular when comparing cases and controls (Lynall et al., 2020). Neutrophils and monocytes are also the immunological cell-types primarily associated with IL-6 and IL-1β (Drevets et al., 2022), while CRP is typically dependent on both cytokines (Howren et al., 2009). Our hierarchical clustering algorithm was applied within a sample including depressed patients only. This can explain why the identified clusters were not associated with monocytes and neutrophils, cytokines levels or depression severity at baseline. This is also consistent with the results of the second cluster analysis, where a one-cluster solution was suggested as best fit. This implies the presence of a relatively immunologically homogenous sample when considering also cytokine levels. In the broader literature, a higher proportion of natural killer cells, CD4+ T-cells and a higher CD4/CD8 ratio has been linked to depressed patients in comparison to healthy controls (Foley et al., 2023; Lynall et al., 2020; Sørensen et al., 2023); which can be seen as a state marker. Interestingly, in our study, the cluster analysis identified these cell types have also been identified as best discriminators within our population of depressed patients, which was then associated with treatment outcome six weeks later. Therefore, these cell-type profiles can be interpreted as

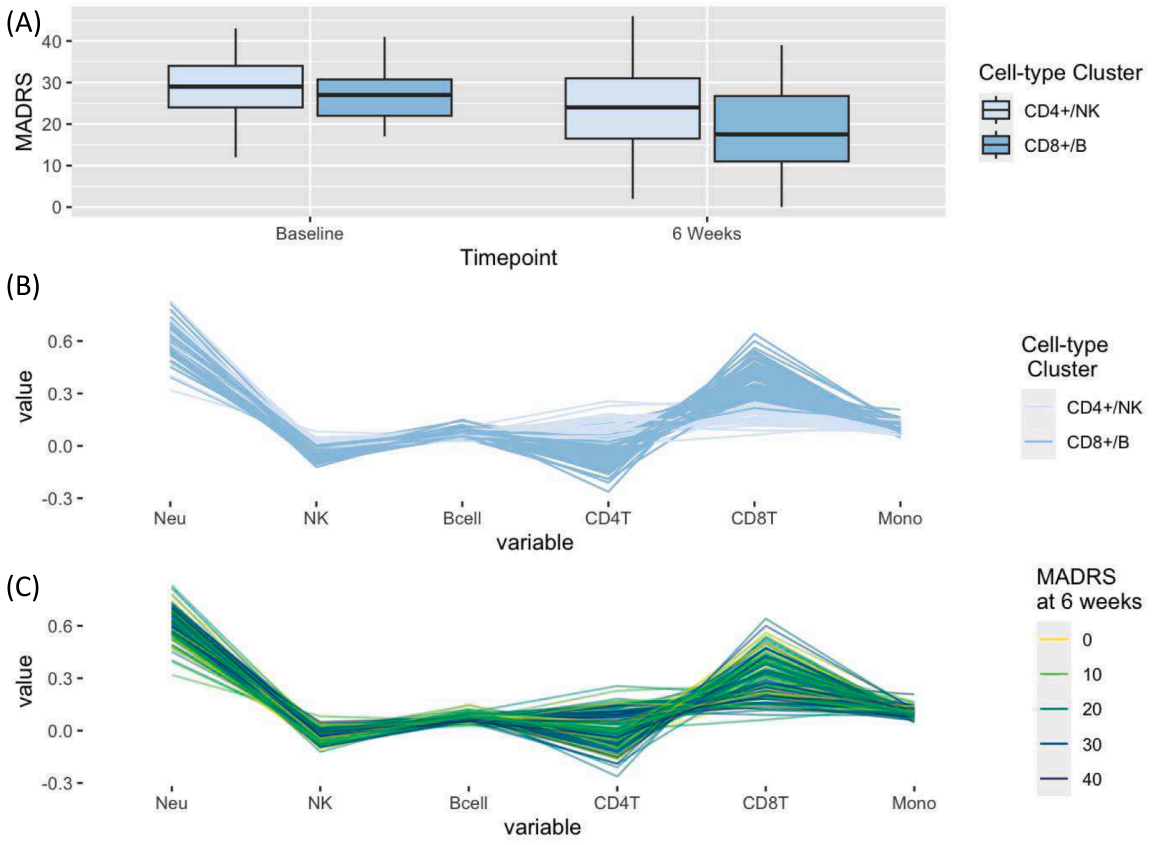

Fig. 3. Estimated Cell-type composition and depression severity at week 6. (A) Both clusters in relation to MADRS at baseline and after 6 weeks. (B) Composition of clusters based on estimated cell-type proportions and (C) reflection of this composition in relation to MADRS at 6 weeks. Value reflects estimated cell-proportion for each of the cell-types.

trait markers too, e.g., regarding likeliness to respond to treatment within a depressed patient population. This might not say so much about inflammation per se, but more about the responsiveness of the immunological system in its mediating capacity in the context of treatment response. As we have no healthy controls available in this proof-of-concept study to use as an additional reference population to answer this question, future research is required to also include healthy controls.

Despite the inflammation-focused recruitment strategy and stratification by hsCRP as a biomarker, hsCRP did not contribute significantly to either cluster analysis. However, our data suggest that, for future research in the context of inflammation and depression, stratification by CD4+/NK proportions versus CD8+/B-cells should be tested for its validity for treatment response prediction.

Based on our first proof-of-concept analysis, we conclude that immunological cell-type composition based on epigenome-wide DNA methylation in blood can be an asset in immunological stratification, specifically for the longitudinal treatment prognosis, e.g., over the course of 6 weeks, as compared to cytokines.

Our study has some limitations. We have no absolute white-blood counts available to compare or validate the DNA-methylation based estimates of cell-types for our sample. Nevertheless, in other samples it was demonstrated that the DNA-methylation based estimates are reliable, being highly correlated with the actual cell-counts (Salas et al., 2018; Titus et al., 2017; Teschendorff and Zheng, 2017). Another limitation is that fractions are estimated. This interdependency can lead to collinearity, which can be an issue for statistical modeling. Using hierarchical clustering, we intend to use the variability among these estimated fractions to overcome this specific issue. Analyses were restricted to six immunological cell-types. This reflects only a small part of all the possible interactions between immunological cell-types and cytokines relevant for the understanding of the complex processes involved in inflammation in depression. A more extensive panel of cell-type proportions and cytokines could be more informative.

In addition, as all our molecular data stem from the baseline assessment, we don't expect our data or results to be affected by the treatment arm patients were assigned to for the rest of the study. Overall, results of anti-inflammatory treatment augmentation are mixed (Simon et al., 2023; Miller and Pariante, 2020; Husain et al., 2020). Nonetheless, cell-type profiles or their immunomethylomic estimates might become valuable early indicators of individuals more sensitive to immunomodulatory treatment modalities.

Age and age-related processes, such as menopause, are also known to affect the immune system and cell-type composition. In particular the age-range between 45 and 55 years is potentially relevant for immunological processes (Nissen et al., 2023). Unfortunately, our sample has no detailed information on women entering (peri-)menopause or other processes typical for this age-range to further investigate their impact on the estimated cell-type composition.

Furthermore, it is well-known that the choice of depression scale affects how the symptom dimensions of depression are represented e.g., by means of selection bias (Fried, 2017). This may affect the interpretation of change in MADRS over time depending on the individual's immunological contribution to the diagnosis of depression in the context of anti-inflammatory augmentation therapy in depression (Wessa et al., 2023). For MADRS, three out of 10 items reflect involvement of somatic symptoms in depression. These items have been shown to be highly correlated with somatic symptom items of other depression scales, e.g., Hamilton Depression scale (HAM-D; 17 items; McIntyre et al., 2006). To better understand the immunological subtype of depression, the

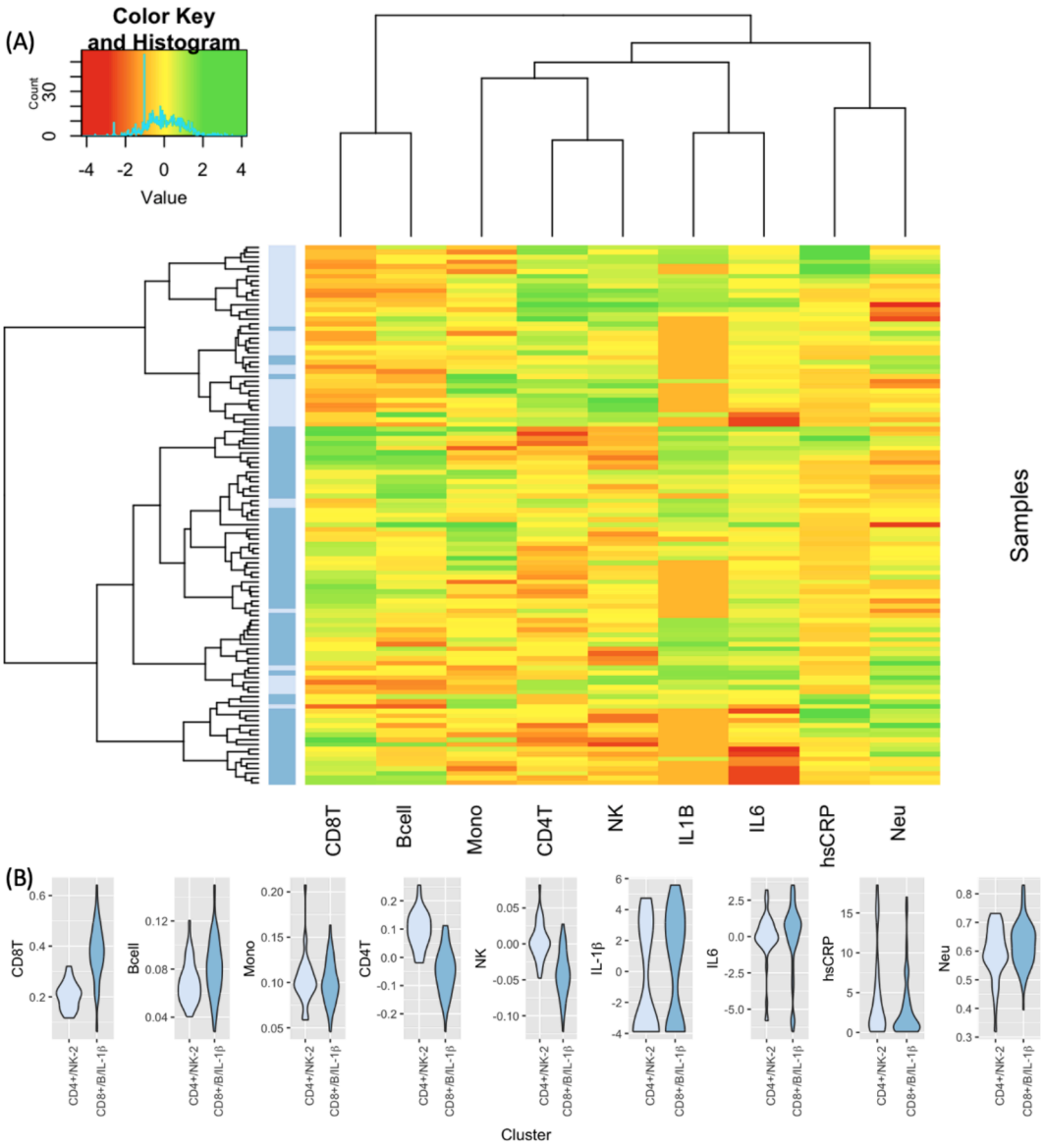

Fig. 4. Result of re-clustering: cell-types and cytokines. Result of hierarchical clustering analysis based on six cell-types, enriched with IL-6, IL-1β, and hsCRP. The blue line (left) reflects the solution of the former hierarchical clustering as shown in Fig. 1 (A). Inconsistencies are the individuals that changed clusters (from CD4+/NK to CD8+/B/IL-1β or from CD8+/B to CD4+/NK-2).

development and validation of a specific scale to differentiate the immune-related subtype of MDD could bring the field forward.

Finally, we expected our sample to be sufficiently powered and diverse. However, the results of the second cluster analysis, with cluster CD8+/B/IL-1β being more highly educated than cluster CD4+/NK-2, shows that groups are not always completely comparable.

As our cell-type estimates rely on epigenomic information, the underlying genomic information remains relevant, including susceptibility to ancestry. We tested this for our sample using the available genomic information which underlined the homogeneity of our predominantly Caucasian sample. However, this also means that more research is needed to extrapolate our results to a more ancestrally diverse population. Also, the association analyses following this second cluster analysis seem to imply that a larger sample size may have resulted in better interpretable results, withstanding our correction for multiple testing. However, our correction may have been too strict, as, of course, the four hypotheses and their analyses are highly interdependent.

Overall, we are optimistic about the use of 'immuno-methylomics' and immunological cell-type profiles based on the cell-type specific DNA-methylation signatures. We showed that the immunological cell-type based profiles are associated with depression severity at baseline and over time. In addition, a hsCRP and cytokine-enriched cluster analysis seemed to be informative for the short-term change of symptoms. Further research with more diverse immunological cell-types and cytokines can be helpful to validate this proof-of-concept study, for the identification and stratification of individuals with an inflammatory depression subtype. Our results suggest that immunological cell-type composition can also play a predicting role in treatment response versus chronicity.

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Evelien Van Assche: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Christa Hohoff:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Ecem Su Atil:** Writing – review & editing, Methodology. **Sophia M. Wissing:** Writing – review & editing, Methodology. **Alessandro Serretti:** Writing – review & editing, Methodology. **Chiara Fabbri:** Writing – original draft, Methodology. **Claudia Pisanu:** Writing – review & editing. **Alessio Squassina:** Writing – review & editing. **Alessandra Minelli:** Writing – review & editing. **Bernhard T. Baune:** Writing – review & editing, Conceptualization, Methodology, Funding acquisition, Project administration, Supervision.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.09.026.

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