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RESEARCH ARTICLE

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Molecular correlates of childhood adversity – a multi-omics perspective on stress regulation

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ABSTRACT

The experience of adversity in childhood can have life-long consequences on health outcomes. In search of mediators of this relationship, alterations of bio-behavioral and cellular regulatory systems came into focus, including those dealing with basic gene regulatory processes. System biology oriented approaches have been proposed to gain a more comprehensive understanding of the complex multiple interrelations between and within layers of analysis. Here, we used co-expression based, supervised and unsupervised single and multi-omics systems approaches to investigate the association between childhood adversity and gene expression, protein expression and DNA methylation in $CD14^+$ monocytes in the context of psychosocial stress exposure, in a sample of healthy adults with ($n=29$) or without ($n=27$) a history of childhood adversity. Childhood adversity explained some variance at the single analyte level and within gene and protein co-expression structures. A single-omics, post-stress gene expression model differentiated best between participants with a history of childhood adversity and control participants in supervised analyses. In unsupervised analyses, a multi-omics based model showed best performance but separated participants based on sex only. Multi-omics analyses are a promising concept but might yield different results based on the specific approach taken and the omics-datasets supplied. We found that stress associated gene-expression pattern were most strongly associated with childhood adversity, and integrating multiple cellular layers did not results in better discriminatory performance in our rather small sample. The capacity and yield of different omics-profiling methods might currently limit the full potential of integrative approaches.

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Introduction

Exposure to stressful and potentially traumatic events in childhood not only causes immediate harm but also represents a trans-diagnostic risk factor for a range of mental health problems across the life span (Sonuga-Barke et al., 2017; Teicher et al., 2022). Furthermore, early adversity and trauma is among most potent and consistent predictors of physical disease and reduced longevity (Snyder-Mackler et al., 2020). These long-term effects and also the stability of early environmental insults has led to the question of "how and when the social becomes biological"; or in other words, how psychosocial risk becomes *biologically embedded*. There is evidence that early adversity during different periods of developmental plasticity induces enduring changes at multiple levels of regulatory systems, and influences social, cognitive, neuronal and emotional development (Lupien et al., 2009).

Among the bio-behavioural systems involved in bi-directional mind-body interplay, alterations of stress response systems, inflammatory immune processes and the stress-immune interface have emerged as likely mediators of the effects of adverse childhood experiences on health outcomes (Heim et al., 2019).

More recently, the sub-cellular realm came into focus. Further, advances in research in animal models and technological developments – especially high throughput *–omics* approaches – provided a framework to investigate the immediate and fundamental mechanisms of gene-environment interplay. Particularly epigenetic modification such as DNA methylation received attention (Houtepen et al., 2016) and RNA dynamics are increasingly being studied (Cole, 2014).

Omics-technology driven access to the entirety of the biochemical landscape of different molecular layers within cells, such as the transcriptome or proteome (Haas et al., 2017), also fuel the realization of system biology approaches in the study of complex traits or environmental influence (Pinu et al., 2019).

System biology approaches emphasize on describing the interrelation of analytes or cell entities to gain insight into

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the complexity of biological processes underlying phenotypic variation and disease risk (Yan et al., 2018). A steadily increasing number of statistical methods allows for the implementation of these perspectives in research targeting more than one molecular layer (Krassowski et al., 2020).

Co-expression network analyses for example reflect a systems approach to single-*omics* analyses and aim to identify network structures formed by highly interrelated analytes. As such, weighted gene-co-expression network analysis (WGCNA) has been used to gain insight into gene co-expression signatures associated with posttraumatic stress disorder (PTSD) (Breen et al., 2018).

Multi-*omics* analyses further advance the idea of molecular systems biology. Such approaches integrate three or more (Krassowski et al., 2020) –*omics* datasets derived from specific profiling methods such as microarrays or mass spectrometry. Although the integration of several large datasets with different modalities impose a certain kind of complexity to the analytical enterprise (Jamil et al., 2020; Pinu et al., 2019) it promises a more comprehensive insight into the molecular machinery unmet by a single-omics based perspective.

While there is overall broad potential, one application of multi-*omics* analyses is the identification of subgroups based on the information embedded within different molecular layers. Such multi-*omics* analyses can be classified into supervised and unsupervised approaches (Krassowski et al., 2020; Subramanian et al., 2020).

Unsupervised approaches are exploratory and data driven. They do not require precise specification of the clinical/experimental group from which the included omics datasets are derived, but may be used e.g. to reveal clusters of participants within the sample. The application of regularized unsupervised multiple kernel learning (rMKL) to gene expression, DNA methylation and miRNA data for example resulted in the identification of subgroups in patients with distinct cancer types, which showed different survival times (Speicher & Pfeifer, 2015). Supervised multi-omics approaches, on the other hand, are hypothesis-driven and seek to identify molecular signatures that best characterize a known and specified clinical/experimental group. Thus, a supervised strategy is particularly suited to identify predictive biomarkers or condition specific molecular processes. *D*ata *i*ntegration *a*nalysis for *b*iomarker discovery using *l*atent *c*omponents (DIABLO; Singh et al., 2019), for example represents a specific supervised approach which has been used to identify molecular signatures predictive of Hepatitis B vaccination response (Shannon et al., 2020)

Taken together, despite increasing insights into adversity-related biological alterations, our current knowledge is fragmentary and mostly based on single levels of analysis. Integrative approaches grounded in a systems biology perspective promise to enhance our understanding of alterations within gene and cell regulatory mechanisms following experience of childhood adversity.

Here, we apply such integrative approaches in the context of acute stress exposure. The sampling across multiple time points takes into account stress-associated hormonal response dynamics and the associated immediate changes on the level of transcriptome and proteome. Given the manifold downstream effects of stress mediators like cortisol, the molecular consequences of a dysregulated stress axis should be become more readily observable after challenging the system compared to unstimulated single-point measures.

Such downstream effects include modulation of immune function, and several studies have observed stress- and adversity-associated changes in particular to proinflammatory signaling. $CD14^+$ monocytes have been identified as the most responsive to socio-environmental conditions (Cole et al., 2011; Miller et al., 2014; O'Donovan et al., 2011) and they further play a crucial role in in immune-to-brain communication processes, carrying proinflammatory signals to the brain (Wohleb et al., 2014; Wohleb & Delpech, 2017) – hence our choice as target cells. Based on cortisol measures, DNA methylation, gene expression and protein expression data derived from $CD14^+$ monocytes, we showcase the application of (i) single-omics co-expression network analysis; (ii) unsupervised and (iii) supervised multi-omics analysis to identify acute stress-associated molecular signatures in adults reporting childhood adversity.

Methods

Study design and context

This study is based on a project investigating long-term consequences of childhood adversity on stress reactivity, which included the collection of transcriptomic, proteomic, and genome-wide DNA methylation data. Initial work focused on individual omics data (see supplements; Frach et al., 2019; Schwaiger et al., 2016; Zang et al., 2021). Here we take integrative multi-omics approaches to further characterize molecular signatures associated with adverse childhood and acute stress experience.

We investigate different biological parameters that are characterized by different time dynamics and change over time. Consequently, this results in differences in timing and frequency of the respective measures. The cortisol stress response is usually measured within a time frame of about 60–90 min to assess stress responsivity and to detect subtle differences in negative feedback regulation. Hence, cortisol measures were taken before and after exposure to the Trier Social Stress Test (TSST), at −45, −2, 1, 10, 20, 30, 45, and 90 min relative to stress exposure (Figure 1A). We calculated participants base to peak cortisol response as an aggregate stress response measure. Blood samples for omics analyses were taken at −45, 45, and 180 min relative to stress exposure to detect stress-associated changes in gene- and protein expression (Darzacq et al., 2007; Hausser et al., 2019). Whereas gene expression changes can be observed at 45 min post stress exposure already, signals at the protein level are more likely to manifest 180 min following stress. DNA methylation levels are assumed to be rather stable in short time frames and were consequently assessed at one time point only (45 min post stress). All omics analyses were performed in isolated $CD14^+$ monocytes (see (Schwaiger et al., 2016; Zang et al., 2021) for more details on the study protocol).

Sample

The original sample ($N = 60$) consisted of healthy adults reporting a history of childhood adversity ($n = 30$) and a matched control group ($n = 30$). Self-reported childhood adversity was

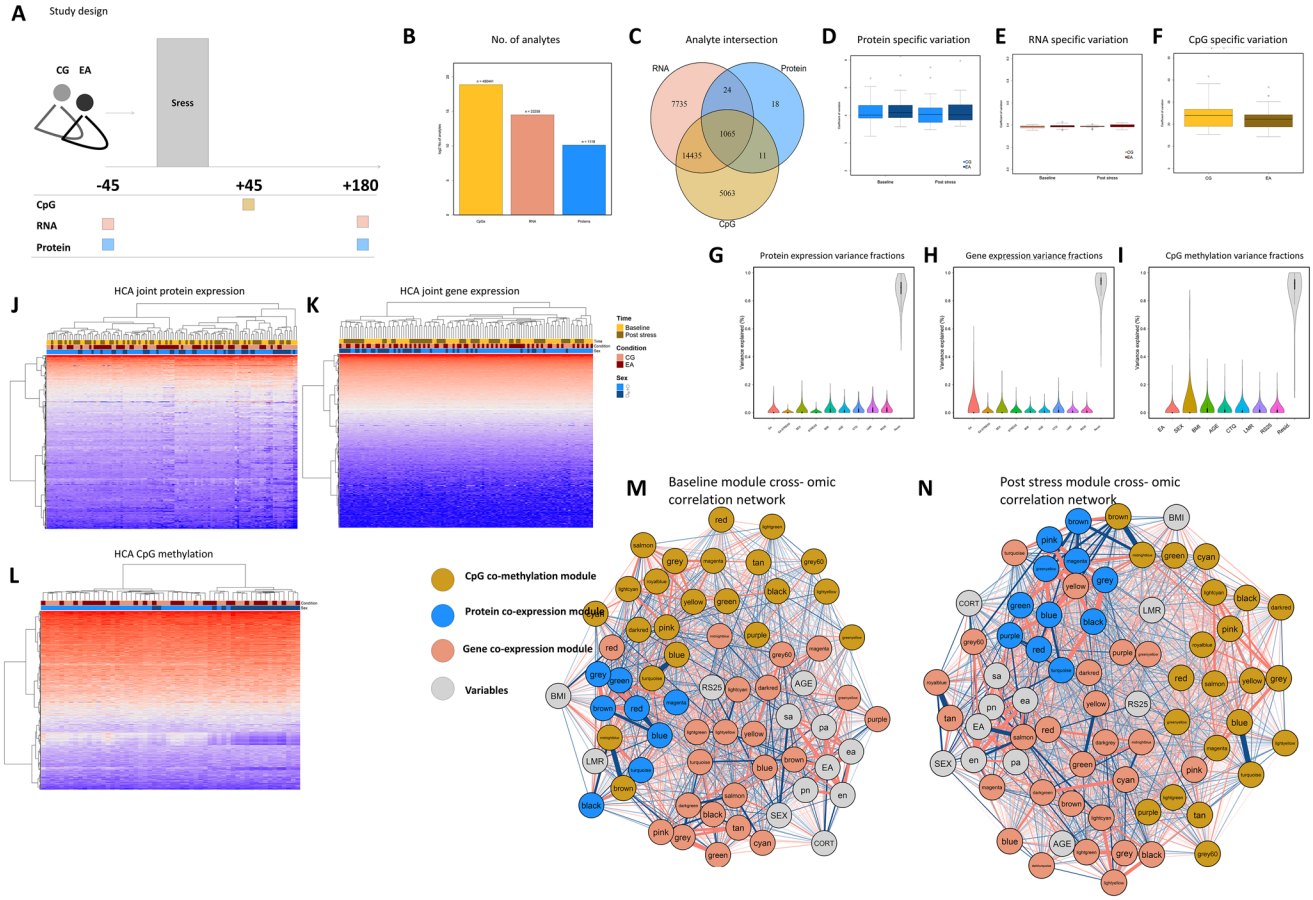

Figure 1. The study design depicts major sampling points of monocytes for transcriptome (RNA = red), proteome (protein = blue) and whole genome CpG methylation (CpG = yellow) analysis in minutes relative to stress exposure (TSST) (A). The number of analytes and intersection of gene names assigned to analytes across omics-datasets is given in (C). Boxplots depict condition specific inter-individual variation (CV) in baseline and post stress gene and protein expression (C and E) and variation in CpG methylation (G). Variance fractions explained by clinical variables within protein (D) and gene (F) expression data and CpG methylation (H) is presented by violin plots. Networks show baseline (I) and post stress (J) correlation (edges) of proteome (blue), transcriptome (red) or CpG methylation (yellow) derived co-expression (co-methylation) modules (nodes). Grey nodes represent variables of interest (see methods).

assessed with the Childhood Trauma Questionnaire (CTQ) followed by a more in-depth interview using the Early Trauma Inventory (ETI). All participants were screened for mental disorders using Structured Clinical Interview for DSM Disorders (SKID I and II) and filled in the Resilience Scale (RS-25). Further, participants' socio-economic status (SES), history of mental disorder, smoking habit, Body-Mass-Index (BMI) and age were assessed. Details of the recruitment and sampling procedures as well as the assessment of further bio-behavioral variables are described elsewhere (Schwaiger et al., 2016). Groups did not differ significantly regarding most of these variables. See supplements for further sample characteristics.

Analyte source

DNA, mRNA and proteins were extracted from CD14^{+} monocytes, a specific immune cell type which has been shown to be sensitive toward social influences (see above). We used immunomagnetic cell separation to isolate CD14^{+} monocytes from whole blood samples. Please refer to the supplements for further information on monocyte isolation.

Omics profiling

DNA methylation levels were quantified with the Ilumina Infinium HumanMethylation450 BeadChip and processed with standard bioinformatic pipelines. Following RNA isolation, genome-wide gene expression profiling was realized using Agilent Whole Human Genome Oligo Microarrays 8x60K V2. Protein was extracted from monocytes using repeated exposure to ultrasonic waves and the application of physical force under precooled and iced conditions. Proteome analysis was realized by Liquid Chromatography tandem Mass spectrometry (see Zang et al. (2023) and supplements for details).

Final datasets

Following preprocessing, we constructed the final sample ($N = 56$) as intersection of participants with valid values on all omics datasets. This final sample included 29 participants with a history of childhood adversity and 27 control participants. Where possible we mapped individual analytes (CpG sites, transcripts, proteins) to gene symbols. Additional variables included group assignment (early adversity vs

control), CTQ scores, RS25 scores, BMI, age, sex, *monocyte* to l*ymphocyte ratio* (MLR) and time of assessment (t0/t1) relative to stress. Please note that there were no major difference between group regarding most of the assessed covariates. As expected, adults reporting a history of childhood adversity reported higher rates of mental disorders in the past compared to the control-group, but had been free of diagnosis for at least 12 months. Participants' endocrine stress reaction was coded as cortisol increase following TSST exposure (baseline-to-peak levels).

Analytical strategy

We first assessed group specific inter-individual variance and analyte specific sources of variance. Subsequently we approached integration of our transcriptomic, proteomic, and CpG methylation data in three ways.

First, we utilized co-expression network analysis to identify modules of co-expressed analytes on the single omics level and subsequently investigated the cross-omics interrelation of level specific co-expression structures.

Second, following an unsupervised pathway to multi-omics integration, we utilized regularized multiple kernel learning for dimensionality reduction (rMKL-DR) and tested how good rMKL-DR differentiates between participants when supplied with gene expression, protein expression, CpG methylation and cortisol response data.

Third, following a supervised pathway to multi-omics integration, we utilized sparse and partial least squares regression discriminant analysis to identify single- or multi-omics derived subsets of analytes that discriminate best between participants with a history of childhood adversity and control participants.

Please note that we followed different analytical pathways here to look into cross-omics relation and multi-omics integration with the main focus to test whether we can retrieve adversity-related meaningful subgroups based on the information embedded within different omics-layers and further variables. We did not, however run classical differential analyses that come with an exhaustive numbers of comparisons and would thus necessitate correction for multiple testing. We reported these kind of corrected analyses within our single-omics studies (Frach et al., 2019; Schwaiger et al., 2016; Zang et al., 2021) but did not correct for multiple testing here, given the study design and the range of similar but unrelated and rather exploratory analytical approaches. Nonetheless, given the number of comparisons, results derived from our correlational analyses (see supplements) might be affected by the multiple-testing problem and we report all our findings with due caution as exploratory and preliminary.

Analysis of stress related and condition-specific interpersonal variation

As a measure of interpersonal variation, coefficients of variation (CV) were calculated as CV = standard deviation/mean on non-transformed raw datasets. Proteome and gene expression specific CV were calculated on globally normalized gene (quantile) and protein expression (LFQ) data, separately for baseline and post stress measurements. DNA methylation specific CV were calculated on basis of m-values. For each analyte and point of measurement CV were determined for participants with a history of early adversity (EA) and control participants (CG) respectively. We calculated Pearson's r to investigate the interrelation between the analyte and time specific CV magnitude and compared variation in baseline and post-stress analyte expression between groups using asymptotic testing as implemented in the cvequality R package (version 0.1.3; cvequality: Tests for the Equality of Coefficients of Variation from Multiple Groups, n.d.; Zang et al., 2021).

Omics specific variance analysis

We used a multiple regression model approach to delineate sources of variation and specifically characterize the extent of variance in CpG methylation, gene expression or protein expression attributable to variables of interest. Two versions of these models were specified, including either participants CTQ total score or participants' scores on the CTQ subdimensions. Both models included participants' age, sex, BMI, LMR, RS25 scores and group (EA/CG). Models designed for the analysis of stress effects additionally accounted for the time of measurement and were extended to include the variable stress (t0/t1) as well as an intersection effect (stress: condition). In all models, sex, condition, stress and the stress:condition interaction were specified as random effects and models were fitted using the R package variancePartition (version 1.22.0; Hoffman & Schadt, 2016).

Co-expression and co-methylation network analysis

As a first approach to intra- and cross-omics relations, we used a combination of weighted correlation network analysis and subsequent hierarchical cluster analysis (HCA) to investigate correlation structures within and across our omics datasets.

First, co-expression and co-methylation network analyses were conducted to examine correlation patterns within the different omics datasets using the application of weighted correlation network analysis by means of the WGCNA R package (version 1.70–3; Langfelder & Horvath, 2008). For the gene and protein expression data, we applied WGCNA to baseline and post stress measures separately to get an idea about potential stress effects on co-expression structures and network topography later on. Subsequently, we calculated module eigengenes, eigenproteins and eigenCpGs (MEs) for all of the retrieved co-expression and co-methylation modules. MEs represent a module's expression profile and were further used to identify modules related to the experience of early adversity or relevant other variables (modules of interest) and to investigate cross-omics interrelation of identified co-expression modules. To visualize and identify clusters of correlated co-expression and co-methylation modules as well as modules' correlation with variables of interest, we applied

HCA and constructed two undirected correlation networks using the R qgraph package (version 1.9; Epskamp et al., 2012). The first network included MEs from baseline co-expression modules and the second network included MEs from post-stress co-expression modules to visualize potential stress effects on cross-omics correlation. Both networks included MEs from co-methylation network analyses as well as variables of interest such as group status, sex or cortisol response to stress (see supplements for details).

An unsupervised pathway to integration; the regularized multiple kernel learning for dimensionality reduction analysis approach

Regularized multiple kernel learning for dimensionality reduction analysis (rMKL-DR) was performed using Locality Preserving Projections (LPP) for dimensionality reduction as suggested by Speicher and Pfeifer (Speicher & Pfeifer, 2015).

To investigate the relative importance of different data types on discriminatory capability, we conducted four different clusters of analyses. Each cluster is characterized by a distinct combination of biological data, and analyses in each cluster were performed three times with slightly varying parameters. The first cluster (M1–M3) contained protein expression and gene expression data. The second cluster added participants' base-to-peak cortisol response to protein expression and gene expression data (M4–M6). The third cluster included gene expression, protein expression, and DNA methylation data (M7–M9). The fourth cluster (M10–M12) integrated all data sets.

For each cluster we ran altogether three analyses with either two, three or five (default setting) projection dimensions and all analyses were performed separately for baseline and post stress data. Across all analyses, the number of neighbors was held at a constant of nine (default setting).

Subsequently, we used multiple regression modeling (see above) to delineate sources of variation of derived subgroups. While we focused our analyses on the question to what extent derived subgroups correspond to participants' group (childhood adversity or control) or the type of adversity experienced (CTQ scores), we also included participants' cortisol response, sex, age and BMI as factors of potential molecular influence (see supplements for details).

A supervised pathway to integration; the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) approach

We utilized supervised discriminant analyses as implemented in the R mixOmics package (version 6.16.3; Rohart et al., 2017) to identify analytes explaining participants group membership (EA/CG). Analyses were performed either on each of the omics datasets separately (gene expression data, protein expression data, or CpG methylation data) using sparse partial least squares regression discriminant analysis (sPLSDA), on gene and protein expression data, or on gene expression, protein expression and CpG methylation data simultaneously using block sparse partial least squares regression discriminant analysis (block sPLSDA) as provided by the DIABLO framework (Singh et al., 2019). All analyses were performed separately on baseline and post stress data. To select the model with the best discriminant performance, we ran each model ten times while increasing the number of selected analytes in steps of 10. Altogether we ran 10 models, and we evaluated model performance on basis of balanced error rate (BER, smaller = better).

Results

Monocyte-derived multi-omics datasets comprised altogether 504,819 analytes including expression values of 23,259 transcripts, 1,119 proteins, and methylation degrees of 480,441 CpGs. 1,065 of these analytes mapped to the same gene name (Figure 1B).

Omics-specific inter-individual variability

The assessment of inter-individual variability across all omics datasets showed highest amount of variation within CpG methylation degrees and lowest variation within gene expression levels (Figure 1C,E,G). Asymptotic tests did not reveal any significant group differences in expression or DNA methylation related variation (Table S4) and there was no significant relation between inter-individual and time specific variability across omics datasets in participants with a history of childhood adversity. In control individuals, variation in post stress protein expression was significantly correlated with variation in baseline protein expression ($r = 0.73$, $p < .001$) and baseline gene expression ($r = -0.39$, $p < .05$) (Table S1–S3).

Omics-specific sources of variance

After controlling for all other variables included in the respective models, early adversity explained altogether only modest fractions of variance in gene expression levels (up to 1.23%), protein expression levels (up to 0.21%) or DNA methylation levels (up 0.11%). Please note that participants' CTQ levels explained some variance in protein and gene expression (between 2.07–2.36%) and DNA methylation levels (up to 1.21%) beyond the variance accounted for by group status (see Table S5–S9). The same was true for the effects of stress exposure and the early adversity by stress interaction, which accounted for 0.22% of variance in gene expression and 0.04% of variance in protein expression (see Tables S8 and S9).

Analyte specific co-expression and co-methylation structures and cross-omics interrelation

Weighted correlation network analysis led to the identification of between 7 and 22 modules harboring co-expressed genes ($n_{t0} = 21$, $n_{t1} = 22$), co-expressed proteins ($n_{t0} = 7$, $n_{t1} = 24$) or co-methylated CpGs ($n = 21$) within the calculated networks (Figures S1 and S2). Uncharacterized analytes were assigned to a grey default module. Gene and protein

co-expression structures identified in baseline networks were largely conserved within the post stress networks (Gene co-expression: Zsummary $M = 20.39$, $SD = 11.83$; Protein co-expression: Zsummary $M = 11.06$, $SD = 4.43$) and WGCNA conducted upon joint datasets revealed 21 gene co-expression modules and 6 protein co-expression modules.

Overall, both within and across layers, identified co-expression and co-methylation modules correlated moderately; however, strong correlations were observed for individual modules (range in baseline network: $r = -0.93$ to 0.93; post stress network: $r = -0.92$ to 0.86; joint network: $r = -0,96$ to 0.83). HCA driven analysis of module correlation indicated stronger within-omics than cross-omics relations (Figure S6). Interestingly, cluster structures showed a negative correlation between most of the co-methylation modules and particular gene and protein co-expression modules. This relation between higher DNA methylation degrees and reduced gene and protein expression became especially evident in baseline and joint data modules. Network structures derived from baseline (Figure 1I) and post stress modules (Figure 1J) validated these observations. Although modules of the same omics origin tended to cluster together within the topology of both pre- and post-stress networks, modules were stronger intertwined in the post stress network – indicated by higher strength and closeness and higher absolute edge weights ($t(4497) = 11.46$, $p < .001$) compared to the baseline network. In both networks, gene co-expression modules showed closest proximity to childhood adversity associated variables (EA or CTQ scores). A closer look at modules' correlation with early adversity revealed the following: First, correlational analyses identified four baseline co-expression modules (two protein modules) positively associated with the experience of early adversity and analytes contained within these modules are functionally implicated in e.g. DNA strand elongation, interleukin-1 beta signaling viral processes and mitochondrial ATP synthesis. Second, following stress there was a larger number of gene ($n = 5$) and protein ($n = 3$) co-expression modules significantly correlated with a history of childhood adversity (Figure S3-S4). Modules of interest harbored analytes involved in e.g. regulation of cellular response to stress, mitochondrial protein processing, viral response or neutrophil activation involved in immune response (Figures S7 and S8). Third, none of the identified co-methylation modules was associated with the experience of childhood adversity (Figure S4A) and we found no relation between protein co-expression and acute stress exposure (Figure S5). For further details, see SI.

An unsupervised pathway to integration. The regularized multiple kernel learning for dimensionality reduction analysis approach

Application of defined models grouped participants in clusters of $n = 2$–10, based on baseline and post-stress omics data and participants' cortisol response as input (Table 1). The groups tended to be larger when based on post-stress omics data. Table 1 shows the extent of the variance explained in the respective clusters by variables of interest using multiple regression modeling. None of the models formed groups that clearly differentiated between participants with a history of childhood adversity and the control group. The largest amount of variance in participants' history of childhood adversity was explained by model 1 (6.14%) based on RNA and Protein expression. Notably, including CpG methylation as data source clearly increased discriminatory capabilities of models particularly when it comes to differentiating participants based on the experience of sexual abuse, sex or age.. For instance, group assignments derived from constructed models explained a substantial amount of variance of CTQ sexual abuse subscale scores (M7 = 29.3%) and participants sex (M12 = 72% of explained variance).

A supervised pathway to integration: the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) approach

We fit multiple sparse partial least squares regression (sPLS) models and DIABLO models (block sPLS) to single omics (gene expression, protein expression, CpG methylation) or multi omics (gene and protein or gene, expression, protein expression and CpG methylation) datasets to investigate the added value of multi-omics integration for the models performance in differentiating between conditions (EA/CG).

Assessment of the model's performance based on BER comparison yielded two central insights. First, single omics gene expression sPLS models clearly outperformed all other models and differentiated better between groups than models based on protein expression, CpG methylation or models integrating two or three omics-datasets. Second, models based on post stress analytes differentiated better between groups than baseline models and this was true for all single or multi-omics models (Figure S9). In tendency, model performance did not increase with the number of analytes and models comprising 20 or 30 analytes of each molecular level reached lowest error rates (see supplements).

The top performing model was the post-stress single omics gene expression model based upon 20 transcripts (Figure 2A). 13 of these transcripts are shared with the best performing baseline gene expression model (Figure S10; Table S10), 14 are shared with in the best performing post-stress gene and protein expression model (Figure 2B) and none were shared with analytes included in the best performing post-stress gene expression, protein expression and CpG methylation model (Figure 2C). Two of the top 20 transcripts corresponded to analytes included in proteomic dataset (HSPA8, H1FX) and 16 CpGs map to the same gene names.

Cluster analyses clearly showed that the top performing post-stress single omics gene expression model differentiates between participants based on two clusters of transcripts (Figure 2A). The first cluster contained transcripts downregulated ($n = 8$) and the second cluster contained transcripts upregulated ($n = 12$) in participants with a history of childhood adversity (Table 2). When supplied to the STRING database, downregulated transcripts formed a small network structure enriched for analytes functionally involved in Chaperone-mediated protein folding (GO:0061077), regulation

Table 1. Variance explained by baseline and post-stress rMKL-models.

			NC		CTQ_TS		CTQ_EA		CTQ_PA		CTQ_SA		CTQ_EN		CTQ_PN		CORT_BP		GROUP		SEX		AGE		BMI	
Model	ML	DIM	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1
M1	RP	2d	3	10	5.35	0	0	3.72	0.16	0	6.78	2.71	0.29	0	7.14	6.66	0	3.37	6.14	0	1.69	5.32	0	0	8.54	0
M2	RP	3d	4	8	4.14	0	0	0	0	0	12.14	6.29	0	0	11.75	2.35	0	7.06	1.75	0	7.33	2.85	0	4.4	5.91	1.78
M3	RP	5d	5	6	5.46	0	0	0	0	0	8.87	9.02	0	0	14.63	5.26	0	6.12	0.4	0	10.62	15.64	0	0	6.58	7.58
M4	RPC	2d	3	4	4.82	0	1.72	0	0	0	6.67	10.1	0	0	3.18	0	0	14.6	3.38	0	5.99	9.94	0	0.38	9.62	0.3
M5	RPC	3d	4	7	2.05	0	0	0	0	0	9.53	0	0	9.26	6.79	13.41	0	0	0	0.1	3.75	17.23	0	0	5.32	7.21
M6	RPC	5d	6	10	3.23	0	0	0	0	0	6.66	0	0	5.3	11.9	10.14	0	3.73	1.2	6.7	11.03	14.88	0	9.89	6.48	7.32
M7	RPM	2d	4	8	6.65	0	0	0	0	1.07	29.33	10.4	0	0	6.48	0	0	13.04	0.42	0	51.59	58.26	6.33	15.62	0.89	5.24
M8	RPM	3d	4	5	2.77	0	0	0	0	0	1.59	10.61	0.49	0	0.99	0	0	16.72	2.96	0	6.68	61.63	0	13.33	6.21	0
M9	RPM	5d	5	6	1.01	5.42	0	3.16	0	9.93	25.42	14.22	0	0	0	1.8	0	16.25	0	0	42.12	65.74	0	15.17	3.99	0
M10	RPMC	2d	2	3	1.43	0	0	0	0	0	0.66	14.32	0	0	0	0	0	7.37	1.13	0	10.14	55.63	0	0	4.79	0
M11	RPMC	3d	2	5	2.77	7.02	0	0.67	0	13.84	1.59	17.59	0.49	0	0.99	0	0	13.33	2.96	0	6.68	52.12	0	7.11	6.21	0.53
M12	RPMC	5d	5	2	1.01	0	0	0	0	0	25.42	15.77	0	0	0	0	0	11.19	0	0	42.12	72.05	0	0	3.99	0

M = Model tested, ML = molecular level (R = gene expression, P = protein expression, M = CpG methylation, C = Cortisol response base to peak response, DIM = rMKL dimensions extracted, NC = number of clusters derived from rMKL analysis, CTQ = Childhood trauma Questionnaire total score (TS = total score, Subdimensions: EA = emotional abuse, PA = physical abuse, SA = sexual abuse, EN = emotional neglect, PN = physical neglect), GROUP = EA vs CG), Values give explained variance in %").

of cellular response to heat (GO:1900034) and protein folding (GO:0006457) such as *HSPA8, HSPH1, CHORDC1 or DNAJA1*. 5 of the 12 transcripts upregulated in participants with a history of childhood adversity formed an interconnected network structure when supplied to the STRING Database. These transcripts include EGR1 and its paralog EGR2 as transcription factors crucially implicated in monocyte to macrophage differentiation and macrophage inflammatory functioning; DUSP2, a member of the dual-specificity phosphatase family which is among others involved in immune cell activation; TSC22D3, a glucocorticoid-induced leucine zipper involved in monocyte anti-inflammatory signaling; and NR4A3, a transcription factor causing the differentiation of monocytes to dendritic cells following exposure to inflammatory signaling

Discussion

In the investigation of gene-environment interplay and the molecular signatures underlying stress related mediation of environmental risk, systems biology-oriented approaches have been proposed to gain a more comprehensive understanding of the complex multiple interrelations both within and between the different levels of analysis. Here, we utilized three approaches of integrating transcriptomic, proteomic, and CpG methylation data to test whether such integration can provide meaningful clues to biological alterations associated with the experience of early adversity, and whether combining multiple levels of analyses enhances group discrimination based on biological measures. Omics-data derived from cells isolated before and after stress exposure, based on the rationale that alterations in stress-associated molecular dynamics should be observable more clearly after stimulation. Following the three outlined paths to integration, we gained the following insights:

First, co-expression network analysis on single-omics level resulted in the identification of protein, gene co-expression and CpG co-methylation modules.

Using the modules eigenvalues, a cross-omics correlational network was constructed, to test associations between modules and associations with variables of interest, such as history of early adversity or the cortisol stress response. Overall, correlation between identified modules was moderate, with occasional substantial correlations, especially between modules of the same analytical origin. The topographical representation of cross-omics interrelation reflected these observations, as co-expression modules of the same analytic origin clustered together. Furthermore, gene expression modules and early adversity clustered closely together, followed by protein modules, which mirrors the observation of significant associations between several gene and protein co-expression modules with childhood adversity. These modules were enriched for transcripts and proteins implicated in immune system related processes, or mitochondrial functioning. Interestingly, none of the co-methylation modules were associated with early adversity and this reflected our previous findings which did not suggest an association between early adversity and alterations in CpG methylation in our dataset (Frach et al., 2019).

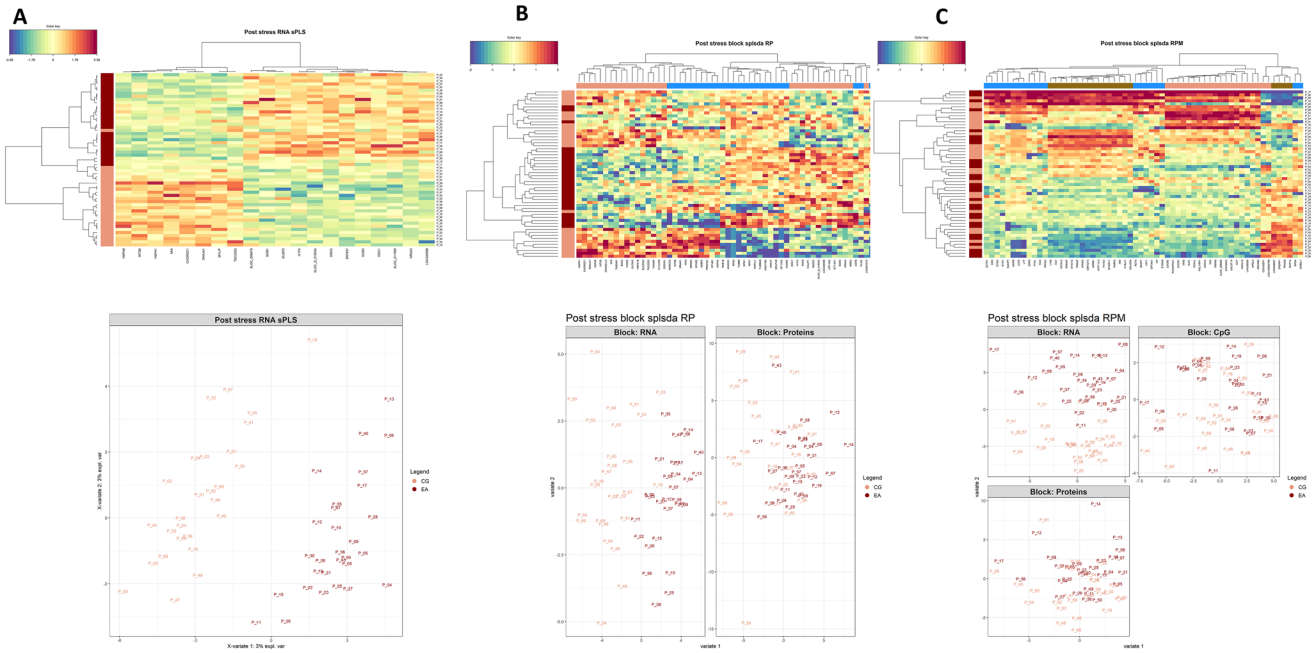

Figure 2. The model with the best discriminant capabilities (A) is based on 20 transcripts derived from CD14^{+} monocytes isolated 180 min after stress exposure. Including post stress proteomic data (B) and CpG methylation data did not result in a variable selection that differentiates better between participants' with a history of childhood adversity and control participants. Heatmaps visualizes clustered analytes (columns) and participants (rows). Row annotations indicate participants' condition (EA = dark red). Scatterplots visualize participants' loadings (EA = dark red) on the first component extracted from analytes of included omic levels (A = RNA, B = RNA and Protein, C = RNA, Protein and CpG Methylation).

Table 2. Analytes of the best performing supervised model (post-stress single omic gene expression).

Characteristic		Overall, $N = 56$	CG, $N = 27$	EA, $N = 29$	Difference[1]	95% $CI_{1,2}$
HSPA8	Mean (SD)	13.26 (0.31)	13.47 (0.24)	13.06 (0.21)	0.42	0.29, 0.54
INTS6	Mean (SD)	8.28 (0.25)	8.46 (0.16)	8.11 (0.19)	0.35	0.26, 0.44
HSPH1	Mean (SD)	11.44 (0.38)	11.72 (0.33)	11.18 (0.20)	0.53	0.38, 0.68
MIA	Mean (SD)	6.88 (0.47)	7.19 (0.38)	6.58 (0.33)	0.61	0.42, 0.80
CHORDC1	Mean (SD)	9.23 (0.26)	9.43 (0.19)	9.04 (0.15)	0.39	0.30, 0.48
DNAJA1	Mean (SD)	12.35 (0.28)	12.57 (0.21)	12.15 (0.14)	0.42	0.32, 0.52
MYLIP	Mean (SD)	11.11 (0.32)	11.32 (0.21)	10.92 (0.29)	0.40	0.26, 0.53
TSC22D3	Mean (SD)	14.59 (0.35)	14.82 (0.31)	14.38 (0.24)	0.43	0.29, 0.58
XLOC_009079	Mean (SD)	8.79 (0.26)	8.62 (0.20)	8.95 (0.20)	−0.33	−0.44, −0.22
EGR1	Mean (SD)	10.13 (0.94)	9.49 (0.64)	10.72 (0.78)	−1.2	−1.6, −0.85
DUSP2	Mean (SD)	6.95 (0.53)	6.59 (0.39)	7.29 (0.42)	−0.70	−0.92, −0.49
H1FX	Mean (SD)	11.03 (0.33)	10.78 (0.27)	11.26 (0.18)	−0.49	−0.61, −0.36
XLOC_12_015034	Mean (SD)	6.43 (0.48)	6.07 (0.36)	6.78 (0.29)	−0.71	−0.89, −0.53
AREG	Mean (SD)	4.63 (1.84)	3.31 (0.79)	5.85 (1.69)	−2.5	−3.3, −1.8
ZNF837	Mean (SD)	8.81 (0.37)	8.55 (0.29)	9.05 (0.26)	−0.50	−0.65, −0.35
EGR2	Mean (SD)	7.54 (1.00)	6.84 (0.57)	8.19 (0.87)	−1.3	−1.7, −1.0
ODC1	Mean (SD)	11.25 (0.39)	10.97 (0.29)	11.50 (0.29)	−0.53	−0.68, −0.37
XLOC_011590	Mean (SD)	5.82 (0.47)	5.48 (0.37)	6.13 (0.32)	−0.65	−0.84, −0.46
NR4A3	Mean (SD)	4.10 (1.76)	2.77 (0.70)	5.35 (1.51)	−2.6	−3.2, −2.0
LOC400099	Mean (SD)	10.67 (0.25)	10.50 (0.20)	10.83 (0.18)	−0.33	−0.43, −0.23

Yet, it is worth mentioning here that especially CpG methylation represents high-dimensional data which converged into a rather imbalanced co-methylation network-structure, and it is unlikely that the comparatively small number of retrieved co-expression modules fully reflect the underlying DNA methylation landscape. We did observe negative correlations between DNA co-methylation and gene co-expression modules, which might reflect parts of the relations between DNA methylation and gene-expression, but the lack of overall inter-omics module interrelations might be explained by the small number of retrieved co-methylation modules.

Second, the joint analysis of different omics datasets with unsupervised and supervised integrative approaches yielded mixed results. In unsupervised analyses, especially those clusters that included three- or four-level data (RNA, protein and DNA methylation, and cortisol) discriminated participants on the basis of sex and BMI. Including DNA-methylation as source of information into the models particularly enhanced the models capability to perform sex-related grouping. This did not come as a surprise, given the known association between sex and distinct DNA methylation patterns. Overall, the different cluster solutions were not strongly related to participant's history of childhood adversity. One exception, however, was the cluster mentioned above, which grouped participants not only by sex but also by the CTQ subscale score "sexual abuse", even after controlling for the influence

of sex on grouping. This may to some extent reflect the severity of experiencing sexual abuse as a specific type of adversity, and may be indicative of adversity-type specific signatures at the molecular level. Altogether, these results suggest that based on the provided omics data, biological meaningful subgrouping can be achieved (here: e.g. biological sex). Yet, while models based on more input layers can in some cases outperform those based on fewer layers, this is not necessarily the case and none of the analyses did sufficiently discriminate between participants with or without reported childhood adversity.

Third, the discriminatory performance of supervised approaches did not profit from multi-omics integration. Including protein-expression and CpG methylation to single layer gene-expression models resulted in decreased capacity to differentiate between participants with a history of childhood adversity and control participants. The model with the best discriminatory performance was based on 20 transcripts measured after stress exposure and this single layer post-stress model outperformed all other protein or CpG methylation-based single layers modules as well. These findings support the results from cross-omics co-expression network analysis and our previously reported results (Schwaiger et al., 2016), which showed a strong relationship between stress-associated changes in gene co-expression and early adversity.

We conclude that – at least in our specific case – integration of different layers of omics data does not necessarily lead to better results compared to in-depth single layer analysis or single-omics based discriminatory analyses. From cross-omics network analyses, we did learn that gene co-expression pattern, and to a lesser degree, protein co-expression pattern, were associated with early adversity. In line with our previous single layer analyses, adversity related co-expression modules were functionally implicated in stress-immune interplay (Schwaiger et al., 2016; Zang et al., 2023) and mitochondrial biology (Zang et al., 2023).

Supervised integrative approaches showed that a set of 20 transcripts differentiated clearly between participants with early adversity and controls. Of note, theses transcripts contained HSP70, a key regulator of glucocorticoid receptor activity, and transcripts involved in monocyte inflammatory function and might be interesting candidates in search for potential biomarkers or childhood adversity associated gene expression signatures.

However, the main take-away is that in our case single layer input outperformed multi-layer input, and we thus did not observe a clear analytical gain through multi-omics integration here. Several reasons relating to theoretical considerations, study design as well as data input or preprocessing might account for these findings.

First, multi-omics approaches are conceptionally intriguing and there is some logic to the assumption that combining different omics levels analytically brings to light the information hidden and spread across layers and thus would result in a deeper understanding of the molecular signatures underlying a distinct phenotype (Sathyanarayanan et al., 2023).

Yet, it is early days for these kind of analyses and currently more and more methods for integration are being developed. Clinical application and evaluation over Time will give a clearer picture of what kind of biological and clinical data benefit from integration using specific methods or a combination of integration approaches.

While our discriminatory analyses did not capitalize on the integration of multiple omics levels, this outcome does not necessarily extend to other approaches utilizing different statistical methodologies with distinct analytical focuses. However, from a parsimony standpoint, a possible finding resulting from a multi-omics approach can be the realization that conducting an integrative multi-omics analysis might not be necessary initially. Such a finding could hold some value, particularly in the context of biomarker development.

Second, although we assessed DNA methylation and protein abundance, our study design will most likely have favored and thus prioritized mRNA expression as the most relevant marker. Given the well-established link between childhood adversity and altered stress system regulation, the rationale of the study was to investigate cellular adaptions following acute stress exposure. The observed adversity-related differences in gene expression might overshadow effects of other layers particularly in light of the number of different analytes (see below) and in light of the chosen time-frame. Time is an extremely relevant factor in the complex and highly regulated process of gene expression and protein synthesis, and different omics-layers follow different time scales when it comes to the realization molecular information. While we clearly had the necessary resolution to detect gene-expression changes following stress exposure (Schwaiger et al., 2016), the correspondence between gene expression and proteins synthesis is often delayed and depending on a cell's needs, so that mRNA might not necessarily become translated into protein at all (Darzacq et al., 2007; Hausser et al., 2019).

Hence, our findings are subject to the boundaries posed by the realized sampling time points, and more fine-grained time series in the context of stress exposure, as well as analyses of time-lagged cross-omics correlations should be incorporated in future studies.

On a more technical note, we used established pipelines to preprocess our data prior to analysis. While these steps are fitted to each layer specifically and prepare the data types for later on analysis, they do not correspond to each other so that some cross-omics signatures might have been lost during these processes. Further, it is important to consider is that our analyses are based on a rather small sample with limited power.

Third, there is an imperfect match in number and coverage between our analytes. Whereas mRNA transcripts cover a great majority of the transcriptome, the 450,000 investigated CpGs cover only a fraction of DNA methylation in the genome. On the level of protein, 1119 analytes included also only represent a fraction of the monocyte proteome. For instance, of the 20 transcripts differentiating between groups only two are represented within the protein dataset, so it is unclear whether these differences would amplify on the actual protein level. To make use the full range of available biological information we supplied all identified analytes to the integrational approaches instead of running analyses on the restricted but intersecting dataset of altogether 1065 analytes that mapped to the same

gene symbol across omics levels. While this approach maximizes information depth, individual omics levels are also weighted more heavily according to the number of analytes they contain, potentially overshadowing the information content of other omics levels. Future studies should aim at integrating "true" genome-wide and proteome-wide data.

To conclude, although we see promise in adopting multi-omics approaches in a systems biology framework to advance our understanding of molecular and gene regulatory processes targeted by early adversity our results were mixed here. We found some evidence for omics- informed clustering, and unsupervised analyses differentiated participants according to biological variables, for example biological sex. Stress related gene-expression pattern were most strongly associated with childhood adversity, and integrating multiple cellular layers within an supervised approach did not result in better discriminatory performance. Our results emphasize the importance of carefully conducted single layer analyses and they as well underscore the importance of analyzing molecular consequences of childhood adversity within the context of stress related physiological activation.

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