Neurotoxic mixture effects of chemicals extracted from blood of pregnant women

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Abstract

Human biomonitoring studies typically capture only a small and unknown fraction of the entire chemical universe. We combined chemical analysis with a high-throughput *in vitro* assay for neurotoxicity to capture complex mixtures of organic chemicals in blood. Plasma samples of 624 pregnant women from the German LiNA cohort were extracted with a nonselective extraction method for organic chemicals. 294 of >1000 target analytes were detected and quantified. Many of the detected chemicals as well as the whole extracts interfered with neurite development. Experimental testing of simulated complex mixtures of detected chemicals in the neurotoxicity assay confirmed additive mixture effects at concentrations below individual chemicals' effect thresholds. The use of high-throughput target screening combined with bioassays has the potential to improve human biomonitoring and provide a novel approach to include mixture effects in epidemiological studies.

We are exposed to hundreds, possibly thousands, of chemicals in our daily lives. The exposome encompasses all environmental factors that influence human well-being throughout life such as lifestyle, chemical exposures and social pressures, and is widely recognized as being relevant to triggering or accelerating disease (*1*). The toxic exposome (*2*) is of particular interest as there is a positive correlation between the increasing number of chemicals produced (*3, 4*) and adverse outcomes such as the increasing prevalence of noncommunicable diseases (*5*), as well as behavioral disorders (*6, 7*). Further, the exposure to endocrine disrupting (*8, 9*) or neurotoxic (*10, 11*) chemicals has been directly associated with impacts on the neurodevelopment of children (*12*).

The chemical exposome is addressed – albeit incompletely – in human biomonitoring (HBM) campaigns, which analyze the chemical burden of different sample matrices such as urine and blood (*13*), breast milk (*14*), or tissues (*15*). Blood or plasma is the preferred matrix for cohort studies because it is more accessible than tissues and more representative for organ exposure than urine (*16*). Most HBM studies focused only on small sets of selected chemicals of concern, such as phthalates, pesticides, bisphenols, or flame retardants, despite the chemical exposome being more complex (*17-19*) and highly variable (*20*). From a toxicological perspective, we can divide the blood exposome into noncontaminants such as endogenous compounds or food chemicals and pharmaceuticals (food & drug exposome), and contaminants, which refers to pollutants such as pesticides, industrial compounds and other man-made chemicals (fig. S1). In cohort studies the exposure to identified and quantified chemicals has been linked to diseases and behavioral changes in different life stages, population subgroups or circumstances (*21, 22*), but the investigated chemicals are likely to constitute only a small fraction of the entire exposome and may not cover all relevant effect drivers.

Cell-based *in vitro* bioassays can be used to characterize the potency and mechanisms of toxic action of chemicals detected in blood and can be linked to disease via molecular initiating events of adverse outcome pathways (*23*). Once potency and mechanistic pathways are better understood, studies in more complex systems may help to

establish causality. It is therefore of paramount importance to develop and define easy-to-perform high-throughput screening assays that consider relevant endpoints and toxicity of chemicals and their mixtures. To date, only a few studies have considered mixture toxicity and even these used only small sets of chemicals, which are often already well-studied and not always representative of the chemical diversity of the exposome (*24*). In a study addressing chemical exposure during pregnancy with language delay in the offspring, a realistic mixture of eight endocrine disruptors was tested in diverse *in vitro* and *in vivo* assays, but the mixture components were not tested individually (*25*). More systematic studies are possible with cell-based assays and one of the largest studies on HBM-relevant mixtures so far has tested 50 mixtures with up to nine components in equitoxic concentrations (*26*). A human-relevant mixture of 29 persistent organic pollutants and perfluorinated compounds was also assessed in the *in vitro* endpoints of neurite outgrowth (*27*) and excitoxicity (*28*). *In vitro* assays can also be used to directly screen for all bioactive components in extracts from human samples (*15, 29*) provided they are high-throughput, sensitive, have low sample volume requirements and are not overly impacted by endogenous chemicals.

This study aimed to investigate how diverse and complex chemical mixtures extracted from the blood of pregnant women act together for the endpoint of (developmental) neurotoxicity. We adapted a robust assay that was developed for water quality assessment with the endpoint of neurite outgrowth inhibition, which is an established key endpoint in most neurotoxicity screening batteries (*30*). Our goal was to characterize not only chemicals detected in blood with this bioassay, but also in designed realistic mixtures and the blood extracts themselves.

Results

We characterized human exposure to neurotoxicants by quantifying a broad spectrum of suspected neurotoxicants in human plasma and measuring the neurotoxicity of detected chemicals and their mixture effects by directly testing the extracted plasma. We extracted 624 plasma samples (approximately 250 µL each) from pregnant women (34th gestational week) in the German “Lifestyle and environmental factors and their influence on the newborn allergy risk” (LiNA) cohort study sampled in 2006-2008 (Supplementary Text S1) (*31*). The samples were sequentially extracted using passive equilibrium sampling (PES) and solid-phase extraction (SPE) (*32*) (fig. S2A). Half of the extract was used for quantitative target screening with gas chromatography (GC) and liquid chromatography (LC) coupled to high-resolution mass spectrometry (HRMS) (fig. S2B), and the other half was dosed into a developmental neurotoxicity assay (fig. S2C). We used mixture toxicity modeling to identify chemical effect drivers and designed artificial mixtures in concentration ratios as they were identified in the plasma extracts. These realistic mixtures were utilized to evaluate how complex mixtures act together and to derive the appropriate mixture effect prediction model for such complex mixtures (fig. S2D).

Complex mixtures of chemicals quantified in plasma samples

1,199 chemicals were selected as target analytes (table S1, Fig. 1A) using three criteria: A) known or likely exposure as found in other cohort studies (e.g., food additives, phthalates, plasticizers, pesticides, and per- and polyfluorinated substances); B) frequently identified environmental contaminants; C) chemicals expected and prioritized

by an *in silico* mixture simulation model for neurotoxicity (*33*) (Supplementary Text S2, table S2). As the Supplemental Text S3 demonstrates, the two-step extraction process was necessary to capture the full range of chemicals with diverse physicochemical properties. Of the 624 plasma extracts, 591 extracts remained for analysis after removal of samples with sequentially high detection frequencies of several analytes (Supplementary Text S4).

All pregnant mothers carried a complex cocktail of chemicals, but the number of quantified chemicals varied largely among samples (Supplementary Text S5). Between 5 and 146 chemicals were detected per subject, with the 10^{th} to 90^{th} percentile ranging from 38 to 79 chemicals. Chemicals were sorted into the six categories, namely industry and consumer goods (ind), pesticides (pest), pharmaceuticals (pharm), personal care products (pers), food and other consumption (food), and endogenous compounds (end), based on entries accessible from PubChem (*34*), the Integrated Chemical Environment (*35*), and/or the Chemicals and Products Database (*36*) (table S1).

The quality controls and calibration regressions of 1,012 of the 1,199 chemicals were robust enough ($R^2 > 0.95$, variability <40%) for automated target screening of the plasma extracts (*37, 38*) (Fig. 1B). 294 of these 1,012 chemicals were identified and quantified in the plasma extracts within the calibration range of 0.1 - 100 $ng/mL_{extract}$ (Tables S3 and S4, Fig. 1B). Measured concentrations were corrected for extraction recovery (*32*) to represent the distributions in the original plasma samples (Tables S5 and S6). 473 chemicals were quantified if concentrations were extrapolated outside the calibration range (Tables S7 and S8, and Tables S9 and S10 after recovery correction). 503 of the 1,012 quantified chemicals were identified in a few of the 41 processing blanks at low levels (table S11). The median concentration of each chemical in the processing blanks was subtracted from the quantified sample concentrations. We assigned confidence scores based on the robustness of retention times (table S1). A score of 1 indicates very stable retention times (shifts <0.05 min for LC, <0.025 min for GC), a score of 2 indicates infrequent and slight shift corrections (shifts <0.1 min for LC, <0.05 for GC), and a score of 3 represents compounds that required shift corrections >0.1 min for LC and >0.5 min for GC. As this paper was based on a broad-spectrum screening analysis and focused on the distribution and mixture assessment of chemicals, we considered all three confidence levels as valid.

The recovery-corrected concentrations of all annotated peaks (table S10) ranged over eight orders of magnitude from 10^{-6} to 10^{2} $\mu mol/L_{plasma}$ (Fig. 1C). Chemicals from industry and consumer goods as well as personal care products were identified with the highest frequencies and were situated in the middle of the frequency distributions. Food-related and endogenous compounds had the highest concentrations. Pesticides were found frequently but at low concentrations. Pharmaceuticals were not frequently identified but were detected at concentrations similar to pesticides. The occurrence of individual chemicals is discussed in more detail in the Supplemental Text S5. We compared the median of the recovery-corrected concentrations in ng/mL_{plasma} within the calibration range from our study (table S5) to concentrations reported in the literature and openly accessible sources such as ExposomeExplorer (*39*), the Human Metabolome database (*40*), and the Blood Exposome database (*41*), and the U.S. National Health and Nutrition Examination Survey (NHANES) in table S12 (*15, 42-64*).

Neurotoxic effects of detected chemicals

The list of 473 detected chemicals (table S10) was matched with the potential mixture risk drivers predicted using the criteria and models defined in (*33*) (table S1) and the top 50 chemicals in terms of concentration and detection frequency in our study to yield a list of 143 chemicals, 121 of which were tested in the *in vitro* neurotoxicity assay (*65*) and 23 chemicals had data from a previous study (*66*).

93 of the 143 chemicals (65%) caused robust inhibition of neurite outgrowth with effect concentrations reducing neurite length by 10% (EC_{10}) ranging from 133 nM (1,2-benzisothiazolin-3-one) to 0.1 M (D-glucitol), thereby covering six orders of magnitude in potency (Supplemental Text S6, table S13). 28 chemicals were specific neurotoxicants (10 chemicals had >10 times lower concentrations to inhibit neurite length than to cause cytotoxicity, and 18 inhibited neurite length but were not cytotoxic), 54 were moderately potent (1-10 times more potent than cytotoxic) and for 11 chemicals the neurotoxicity occurred below cytotoxic concentrations and was an indirect effect of cytotoxicity.

Plasma extracts caused concentration-dependent neurotoxicity

The compound narciclasine served as the reference compound for the endpoint of neurite outgrowth inhibition (*67*) (fig. S5). The samples were tested in an 11-point serial dilution with the highest tested relative enrichment factor (REF) of 0.26, which corresponds to a 1:4 dilution of the original plasma sample. None of the plasma extracts caused cytotoxicity but the concentration-response curves for neurite development ((*68*), example in fig. S7) of 535 (85.7%) of the 624 plasma samples were robust enough to be able to derive EC_{10} values (table S14). 19 samples (3.0%) were split for PES and SPE extracts. 21 extracts (3.4%) were identified as inactive, while up to 49 extracts (7.8%) were active but had a nonconfident concentration-response curve. 502 extracts were valid in both chemical and effect analysis.

The 502 raw bioanalytical equivalent concentrations [BEQ_{bio} (eq. S1), in units of nanograms of narciclasine per milliliter of plasma] were log normally distributed and ranged over three orders of magnitude (fig. S8A). 22 out of 41 processing blanks were inactive or had inconclusive concentration-response curves, whilst the other 19 were at the lower end of the sample BEQ_{bio} distribution. It was not possible to extrapolate effects back to a REF of 1, since the concentration-response curves of most plasma extracts formed plateaus at lower REF (for example fig. S7) and linearity of concentration-response curves can only be assumed until a maximum of 30% effect (*69*). The strong inhibition of the neurite development by the sample extracts cannot be caused by chemicals alone, otherwise cytotoxicity would also have to be observable as most chemicals caused cytotoxicity and effects (table S13).

To differentiate between chemical and natural matrix-driven effects, pooled human plasma samples were stripped with activated charcoal to remove all adsorbable organic chemicals before extraction. The concentrations after spiking were quantifiable for 830 of the 1,199 chemicals. Charcoal-stripping removed 738 chemicals by >90%, 60 chemicals by between 50% and 90% and 15 chemicals by between 10% and 50% and 20 chemicals by <10% (table S1). The charcoal-stripping was considered sufficiently depletive as for all groups of chemicals with diverse physicochemical properties >90% of the chemical burden was removed.

The distribution of BEQbio of the charcoal-stripped plasma extract (BEQ_{matrix}) of the charcoal-stripped plasma overlapped with the lower half of the distribution of BEQ_{bio} of the plasma extracts (fig. S8A, table S15). Hence, the BEQ_{bio} of the plasma extracts (table S14) were matrix-corrected by subtracting the median BEQ_{matrix} of the charcoal-stripped plasma samples (table S15) to yield the final matrix-corrected BEQ_{bio} of the sample (fig. S8B). This matrix correction includes the correction by the processing blanks because the charcoal-stripped plasma underwent the same extraction process as the plasma samples.

Effect modeling of mixtures of chemicals detected in the plasma extracts.

We transformed the concentrations of the 75 neurotoxic single chemicals (C_i) identified in at least 10 of the 591 plasma samples into effect-scaled bioanalytical equivalent concentrations $BEQ_{chem,i}$ (eq. 2) by multiplying C_i (table S3) with the relative effect potencies REP_i (table S13), which were defined relative to the reference compound narciclasine (eq. 3). These $BEQ_{chem,i}$ (table S16) were summed up to yield the group BEQ_{chem} (eq. S4 and table S17).

The two most commonly applied mixture toxicity models are “concentration addition” for compounds with similar modes of toxic action, and “independent action” for chemicals with strictly dissimilar modes of toxic action (*70, 71*). It was previously argued that this distinction is unnecessary for realistic (very low) dose levels (*72*). In fact, below 10% effect, the concentration-response curves in *in vitro* bioassays are typically linear (*73*) and both mixture models converge to a common model that is equivalent to summing up the $BEQ_{chem,i}$ of all quantified chemicals (eq. 4) to represent the chemical-driven mixture effect BEQ_{chem} (*69, 74*). BEQ_{chem} refers to the known portion of the effect explained by the detected chemicals and is therefore often termed as the “tip of the effect iceberg” (Fig. 2A).

BEQ_{chem} was further divided into the contributions of the mixtures of individual chemical categories (table S17): industry and consumer goods BEQ_{ind}, pesticides BEQ_{pest}, pharmaceuticals BEQ_{pharm}, personal care products BEQ_{pers}, food and other consumption BEQ_{food} and endogenous compounds BEQ_{end}. Categorization followed the earlier grouping of chemicals and had no implications for grouping of mixture interactions as the category was not associated with the mode of action but solely the usage. The categories are also associated with chemical risk assessment regulation because in Europe industrial chemicals are regulated by REACH (*75*), pesticides by the EU Regulation No 1107/2009 on Plant Protection Products (*76*) and pharmaceuticals by the European Medicines Agency (*77*).

In the 591 tip-of-the-iceberg mixtures (table S17), the endogenous compounds had the highest concentrations in the extracts, followed by industrial and food-related chemicals (Fig. 2B). The distributions of REP_i covered a similar range of four orders of magnitude for all chemical categories and over half of the bioactive chemicals were assigned to the group of industrial chemicals (Fig. 2C). Between 1 and 44 quantified bioactive chemicals contributed to the mixture BEQ_{chem} (table S17). BEQ_{chem} of the individual chemical groups ranged over 8 orders of magnitude (Fig. 2D, table S17). The industrial chemicals had on average the highest contribution to the explained effects (Fig. 2E). One to 10 chemicals were sufficient to define 90% of BEQ_{chem} (table S18). Although each mixture was dominated by 10 or fewer chemicals, a total of 47 different chemicals

were contributing. The BEQ_{chem} explained on average 0.21% (0.0001 to 13.9%) of the matching BEQ_{bio} (Fig. 2F, table S18).

Chemicals with the highest contribution to *in vitro* neurotoxicity were the food additive lauryl gallate, the oxidant 2,6-di-tert-butyl-1,4-benzoquinone, and quaternary ammonium biocides (e.g., benzyldimethylhexadecylammonium, hexadecyltrimethylammonium, benzyldimethyltetradecylammonium, and lauramidopropylbetaine). The biocide 1,2-benzisothiazolinone, the oxidant 2,6-di-tert-butyl-1,4-benzoquinone, the synthesis intermediate 1-naphthol, the flame retardant tris(chloropropyl)-phosphate (TDCPP), and fatty acids (azelaic acid or palmitoylethanolamide) also frequently contributed to the predicted mixture effects (table S19).

Adopting a more realistic but less certain scenario, we performed the same iceberg modeling with concentrations outside the calibration range (fig. S9). This led to an increase in the number of chemicals identified in the plasma extracts and active in the bioassay from 75 to 92, which also resulted in an increase in concentrations (table S9, fig. S9A) and BEQ_i (table S20). Between 1 and 57 quantified bioactive chemicals contributed to the mixture BEQ_{chem} (table S21). The contributions to BEQ_{chem} of the pesticides, food-related chemicals and personal care products increased (fig. S9D), but they remained below the contribution by industrial chemicals (table S21). One to 12 chemicals were sufficient to explain 90% of the BEQ_{chem} but the majority of BEQ_{bio} was $BEQ_{unknown}$. The mean explained percent of BEQ_{bio} increased to 0.31% with a maximum of 10.4% (fig. S9G, table S22).

Including extrapolated concentrations, the fluorinated insecticide flubendiamide, caffeine, and the anti-inflammatory drug ibuprofen identified as important additional contributors to the mixture effects (table S23).

To illustrate what effects to expect from the individual quantified chemicals and chemical categories in the original plasma samples, the BEQ_i were back-calculated to $effect_i$ (in %) triggered by chemical i at the concentration detected, i.e., at a REF of 1 (eq. 6). As these effect levels were in the linear portion of the concentration-response curve below 10% effect, the effects (Tables S17 and S21) can be summed up in the same way as the BEQ_i (table S16 and table S20). Only in a few plasma samples did the effects of all detected chemicals exceed a total effect of 1% with a mean of 0.5% (fig. S10A). If extrapolated concentrations are included, the average effect at REF 1 increases for the combined BEQ_{chem} up to 1% total effect (Fig. 3A and fig. S10B). The effect of the total mixture was not explained by one category alone, but all chemical categories and within each category diverse individual chemicals contributed to the effect (Fig. 3A).

Designed mixture experiments in realistic concentration ratios.

To confirm from another angle that the assumption of concentration additivity between all chemicals and between the different chemical categories was correct, we designed realistic mixtures based on the concentration ratios identified in the plasma extracts and ran them in the same neurotoxicity assay as the samples at concentrations that would reach 30% of the simulated effect. The volumes that can be pipetted are the major limitation when recreating complex real-life mixtures. We selected the most diverse mixtures and narrowed it down to physically feasible mixtures, based on our pipetting equipment. Some mixtures also started to precipitate as the soluble concentration

threshold was exceeded when resolubilized in assay medium. Accordingly, this restricted the number of suitable mixtures for experimental testing. By several rounds of simulation and optimization, the highest number of mixtures that we could prepare and test with DMSO stocks was 63 out of 591 plasma samples that were (bio)analytically characterized (table S24).

61 out of the 75 tip-of-the-iceberg chemicals (table S25) were used to design 63 mixtures with 4 to 28 chemicals in DMSO (table S26). The chemicals covered a wide range of hydrophobicity with $D_{lip/w}$ ranging nearly 8 orders of magnitude and REP_i ranging over 3 orders of magnitude (Fig. 3C). These mixtures were not ideal because of the background effect of the DMSO. DMSO reduced the neurite length above volume fractions of 0.05% (fig. S11). Such a low DMSO content was not achievable for the experiments. For practical reasons, we had to work with a constant background of 0.1125% DMSO. This resulted in inactivity of 25 of the 63 mixtures ((*68*), table S24). The concentrations (Fig. 3D) and mixture effects (Fig. 3E, table S25) were mainly dominated by industrial and personal care products. Despite the issues with the DMSO background, the agreement between prediction of concentration addition and experimental mixture effects was very good. Measured $BEQ_{bio,mix}$ of the 27 mixtures were within a factor of two to the predicted $BEQ_{chem,mix}$ and 11 mixtures agreed within a factor 10 (Fig. 3F, table S26).

The single chemicals had been dosed solvent-free, by preparing the stock solutions in methanol and removing the methanol after aliquoting to glass dosing vials with a gentle nitrogen stream. Effects caused by the methanol blank only occurred at the highest concentrations and were not reproducible ((*68*), table S24). This method was also applied for the second mixture experiment, where 15 mixtures were designed using 45 of the 75 tip-of-the-iceberg chemicals with 4 to 17 chemicals (Tables S27 and S28). The diversity of the chemicals remained high (Fig. 3G) and comparable to the DMSO mixtures, but the number of mixtures was limited for practical reasons. Endogenous compounds had the highest concentrations in these designed mixtures (Fig. 3H), but effects $BEQ_{chem,mix}$ were dominated by industrial and personal care products (Fig. 3I, table S27). 14 out of the 15 mixture $BEQ_{bio,mix}$ were within a factor of 2 to the predictions $BEQ_{chem,mix}$ and only one $BEQ_{bio,mix}$ was within a factor of 10 (Fig. 3J and table S28).

Discussion

Chemical diversity of mixtures in plasma.

The unbiased extraction method combined with an unsupervised automated target screening workflow enabled the quantification of hundreds of analytes in hundreds of samples, which is an important aspect for the analysis of complex mixtures in human biomonitoring. Previously studied contaminants like phthalates, bisphenols, flame retardants, pesticides, and perfluorinated compounds were included but the focus was on complex mixtures of chemicals from diverse sources.

Plasticizers, perfluorinated compounds and other chemicals occur ubiquitously and can be introduced by the sample tubes used for storage or extraction and analysis processes (*78, 79*). We excluded chemicals such as tris(1,3-dichloroisopropyl)phosphate, bisphenol A, lauramidopropylbetaine and triethylene-glycol-monobutyl-ether, which were also found in some of the processing blanks (table

S11). Even if introduced via storage or the analysis procedure (*78*), those chemicals would be present in the samples and contribute to the mixture effects in the bioanalysis.

Despite the limitations of the broad analytical screening method, the overall distributions of concentrations in plasma are plausible, with the highest concentrations belonging to endogenous and food-related compounds as well as chemicals from personal care products, followed by industrial chemicals and pharmaceuticals, while the pesticides occurred in the lowest concentrations (Fig. 1B). The external calibration covered concentrations of 0.1 – 100 ng/mL$_{extract}$, and 294 chemicals were present in concentrations within this range (Fig. 1B) that translates into recovery-corrected concentrations in the plasma from 0.1 nM to 1 µM (Fig. 1C). However, the total number of quantified chemicals increased from 294 to 473 chemicals if extrapolated concentrations outside the calibration range were included. This is prominently visible for the pesticides and pharmaceuticals, where the total number of annotated chemicals increased significantly if extrapolated concentrations were included (Fig. 1B).

The quantification of 294 to 473 chemicals in plasma is a major improvement compared to the usual targeted analysis focusing on only few selective analytes (*80*). Even suspect screening studies are often limited to fewer than 100 identified chemicals but do not yield quantitative data (*81*). The automated target screening (ATS) workflow had an average error in quantification of 30% which was higher than for most targeted approaches, but allows the unsupervised quantification of hundreds of analytes simultaneously (*37*). Still, ATS has an average and fully unsupervised peak annotation accuracy of 82%, meaning that to some degree false positive and negative peak annotations will be introduced. The recent advances in nontargeted analysis and suspect screening allow the identification of thousands of chemical features . The exchange of chemicals that did not yield satisfactory calibration curves or that were not detected in any of the samples with suspects identified by nontarget screening studies has the potential to further increase the number of detected and quantified chemicals using the proposed strategy.

The distributions of pharmaceuticals (median 10 nM) and endogenous compounds (median 60 nM) were lower by a factor of ten to one hundred than in the literature review of Rappaport et al. (*16*). As summarized in table S12, 15 of the 21 compared chemicals were in a similar concentration range, one had inconsistent literature data and four were significantly lower than their concentrations reported in literature, but these were mainly pharmaceuticals and endogenous compounds (e.g., ibuprofen, azelaic acid). It is likely that pregnant women are more cautious that the general population with their consumption of painkillers such as ibuprofen. Further, the pharmaceuticals were the group of chemicals with the highest frequency of confidence scores of 3, indicative of less confident annotation (table S1).

Regarding industrial and consumer product chemicals, exposure to chemicals can and will differ between populations of different socioeconomical background and lifestyles. Hence, differences between sub-populations, for example the slightly elevated levels of mono-iso-butyl-phthalate, DINCH, or PCB-138, are considered within a tolerable range of variability.

While the inclusion of extrapolated concentrations shifted the pesticides into a more realistic distribution, the pharmaceuticals remained low but literature data were also

partially from pharmacokinetic studies with prior intake (*16*). In addition, the extraction method has a tendency for lower recovery of cations, which also mainly affects the group of pharmaceuticals (*32*). Recovery-corrected and extrapolated concentration values were used to mimic a more realistic mixture scenario for most of the chemicals. However, for other analyses such concentration values should be used with caution, as concentrations of chemicals corrected with very low recoveries such as some permanent cations can increase to very high and more unlikely levels close to 100 µM (table S10).

The group of industrial chemicals is a very diverse set of chemicals and comprised nearly a third of the 1,199 chemicals (Fig. 1A). Hence, it is plausible that in total the industrial chemicals are identified as the most frequent group throughout the plasma extracts (Fig. 1B).

As the detected chemicals covered a very broad and diverse set of physicochemical properties (fig. S3) and bioactivity (fig. S4C and D, table S13), the need for the broadest possible extraction of chemical mixtures was confirmed. The PES extracts covered the concentration range of all chemical groups (fig. S4A). The diversity of chemicals is high with hydrophobicity represented by $D_{lip/w}$ ranging over 8 orders of magnitude. Toxicity increased with hydrophobicity, demonstrating the toxic relevance of hydrophobic chemicals and the importance of including them in HBM strategies (fig. S4). Some hydrophilic chemicals acted specifically: Five of the 19 chemicals with a specificity ratio [inhibitory concentration leading to 10% cytotoxicity (IC_{10})/EC_{10}] >10 had a $D_{lip/w}$ below 50 (fig. S6, table S13).

High-throughput neurotoxicity assay adapted to plasma extracts

The neurite outgrowth inhibition assay used in this study had been designed and applied for water quality testing, for which matrix interferences were evaluated (*66*). Plasma is a more complex matrix. Although PES excludes charged chemicals, proteins and carbohydrates (*82*), PDMS can extract lipids. This does not occur to a measurable degree but is in principle possible and will disturb the bioassay performance (*83*). SPE is also a cleanup and extraction method but, as the experiment with charcoal-stripped plasma showed, coextracted some bioactive matrix, even if the matrix does not dominate the mixture effect. Hence, if human samples are dosed in *in vitro* bioassays the contribution of matrix effects needs to be considered (Fig. 2F).

Peptides coextracted by the SPE are likely causes of matrix effects. Albumin is the most abundant small protein in plasma. ~~Direct dosing of plasma to the differentiated SH-SY5Y cells resulted in immediate cell death (data not shown).~~ The extraction of plasma with PES and SPE apparently removed substantial matrix effect, but the endpoint of neurite development was still very sensitive to the presence of undefined organic materials from human samples. Neurite development is a delicate and nonspecific endpoint based on morphological changes of neuronal cells and this susceptibility to undefined organic materials may not be the case for other endpoints, e.g., reporter gene assays indicative of receptor-mediated effects. It is recommended to always include chemical-free matrix samples as controls, e.g., charcoal-stripped plasma, to account for such effects.

Besides neuronal transmission and cell migration, the endpoint of neurite outgrowth inhibition is only one, yet common, *in vitro* endpoint for neurotoxicity. Other results may

be achieved and different effect drivers identified if a different test system was used. This indicates the need for either multiplexed assays which combine several endpoints or *in vitro* test batteries to allow a broader coverage of toxic mechanisms (*30*).

Mixture effect drivers.

The designed mixtures represented the distributions of chemicals detected in plasma at real concentration ratios. Endogenous and food-related chemicals had visibly higher concentrations in the mixtures (Fig. 2B and 3B) but the industrial chemicals were the main effect drivers (Fig. 2D). There is some bias because over half of the chemicals with experimental bioactivity data were industrial chemicals (Fig. 2B). This is a drawback of the study design which aimed to focus on xenobiotic and exogenous chemicals and had only few active chemicals from the food-related and endogenous spectrum (Fig. 2A). Non-contaminants play a role for mixture toxicity because the distributions of REP_i of all chemical categories were similar for neurotoxicity (Fig. 2B).

The *in silico* prioritization helped to narrow down the candidate chemicals of concern from 1,199 potential chemicals to 104 relevant chemicals of which 30% were successfully quantified in human plasma and confirmed to be contributing to mixture effects. As 72-85% of all bioactive chemicals turned out to be baseline toxicants (fig. S6), the concern that the *in silico* method would underestimate the mixture effects was superseded by the ability to fill many data gaps with baseline toxicity predictions and to prioritize chemicals for testing that had previously been overlooked, including bioactive endogenous compounds. Inclusion of baseline toxicity predictions resulted in a prioritization of several highly hydrophobic endogenous compounds such as squalene, oleic acid amide and several fatty acids (table S1). Experiments confirmed this prioritization because endogenous compounds mainly contribute to the mixture effect via fatty acids and fatty acid amides (table S19, table S23). However, due to the low solubility of superhydrophobic chemicals in assay medium, some did not elicit any effects up to the highest soluble concentration. It is known that some chemicals have too low solubility to trigger baseline toxicity on their own but can contribute to mixture toxicity (*84*).

The portion of mean explained effects, 0.21% or 0.31%, was rather low, but this is a common observation for mixture modeling of apical effects in complex environmental samples (*85*). The discussed sensitivity to natural matrix also negatively affects the interpretation of mixture effects, as the natural background artificially elevates sample effects. Further, it could imply that even this broad set of analytes is not covering the most important effect drivers. Whilst the category of industrial chemicals clearly dominated the mixture effects, the effects throughout all mixtures were widely distributed over the chemical categories and individual chemicals (Fig. 2C). This shows that even this small fraction of the observed effect has a very high diversity and complexity of contributing chemicals, reinforcing the need for mixture toxicity of broad sets of chemicals to be considered in human risk assessment and to be included in epidemiological studies. We only assessed the effects of organic chemicals as this group of contaminants represents a large number of unknown chemicals. Metals are also well defined neurotoxicants, but were not covered by our extraction methods. Studies combining both mixture effects of organic chemicals as well as metals may be an interesting approach for future studies.

Concentration additivity and predictability of mixture effects.

One important observation in our study was that the explained effects were not solely defined by one chemical group but by a diverse combination of all chemical categories (Fig. 2E and 3A). Intuitively one would doubt that such diverse chemicals act concentration-additive in mixtures. This is not the case. By designing 78 complex artificial mixtures with 4 to 28 components composed of 45 to 61 identified chemicals, we clearly showed that concentration addition is a suitable mixture prediction model.

Concentration additivity also demonstrated the so-called 'something from nothing' effect, where the mixtures triggered effects even if the individual chemicals would be considered inactive on their own. If all chemicals including the extrapolated concentrations were considered in the mixture prediction, the average of the overall effect of detected chemicals would reach up to 1% total effect (fig. S10). This is still not an experimentally detectable effect in *in vitro* assays, but it also stems only from a fraction of the present chemicals. It remains unclear how quantitative *in vitro* to *in vivo* extrapolations (QIVIVE (*86, 87*)) perform for mixtures, but 1% effect could be considered as a threshold above which concerns are warranted, especially if in the future the number of detected and quantified bioactive chemicals further increases.

This study demonstrates that complex mixture effects in human blood at low effect levels are predictable and that mixture toxicity should be urgently included in human health risk assessment. The big question of which chemicals should be included in mixture risk assessment and how to set up common assessment groups (*88*) according to mode of action is almost futile because concentration addition and independent action are mathematically equivalent below 10% effect (*69*). Chemicals that merely act as baseline toxicants can contribute to mixture toxicity, albeit with a low REP_i. Even without invoking interactions of chemicals in mixtures, mixture effects are more important than effects of individual chemicals. In 5% of *in vitro* mixture studies synergistic and antagonistic mixture effects have been observed (*89*). The majority of those studies were, however, looking at binary mixtures at high concentrations, and the influence of such synergistic or antagonistic mechanism are often linked to specific groups of chemicals (*90*). The concept of concentration addition applies independent of the groups of chemicals and also for complex mixtures, as clearly demonstrated in this study. We agree with Martin et al. (*90*) that synergism and antagonism should be accounted for if susceptible groups of chemicals such as pesticides or metals are in focus, but otherwise additive models are sufficient, especially for complex mixtures of organic chemicals.

Conclusion

Nontarget screening points to thousands of chemicals in a blood sample (*91*) and the simulation of the blood exposome by Rappaport et al. (*16*) also estimated that hundreds of chemicals are present. We were able to quantify hundreds of chemicals in hundreds of human plasma samples using unbiased extraction methods and automated target screening. This approach extends the chemical space of compounds screened in human biomonitoring dramatically and is aiming towards approaches that target the diversity of mixtures in humans instead of individual pollutants in epidemiological studies. The robust *in vitro* neurotoxicity assay based on the development of neurites in differentiated cell lines allowed sufficiently high throughput to screen hundreds of

extracts and hundreds of chemicals and designed mixtures. Detection of neurotoxic mixture effects *in vitro* indicates the presence of neurotoxic chemicals. The next step is to study if their presence is associated with adverse neurodevelopmental health outcomes in epidemiological studies.

If both exposure and effect data are available, the presented approach of summation of BEQ can be extended to other toxicological endpoints of interest and help in prioritizing chemicals of potential concern and eventually identify mixture effect drivers. This approach clearly requires high-throughput screening assays that are associated with human-health relevant endpoints and allow the characterization of hundreds of chemicals. Ideally these assays should also be applicable to testing blood extracts directly. This was only partially successful in the present study and will require further bioassay refinement.

The increasing presence and abundance of bioactive anthropogenic chemicals is unprecedented in human history. The methods presented here give us the tools to investigate complex mixtures and their relevance for human health and risk assessment in a practical way.

Figures

Fig. 1. Compound selection and distributions of identified chemical categories. **(A**) 1,199 chemicals included for target screening. The mix consisted of compounds that were frequently identified in environmental samples, but also chemicals that were prioritized by an *in silico* mixture prioritization workflow for the endpoint of neurotoxicity. **(B)** Distribution of categories of chemicals included and identified in the target screening of the plasma extracts. **(C)** Quantiles of all quantified concentrations in 624 plasma extracts, with grey extensions of distributions to extrapolated concentrations. Pie charts represent the frequency of chemicals in the corresponding categories.

Fig. 2. Iceberg modeling of plasma extracts.
(A) The whole iceberg is defined by the bioanalytical equivalent concentrations (BEQ) of the sample extracts [BEQ_{bio} (eq. S1), in units of nanograms of narciclasine per milliliter of plasma]. BEQ_{bio} can be divided into the explained tip of the iceberg BEQ_{chem}; the portion of matrix effects BEQ_{matrix} (derived from charcoal-stripped plasma); and the unknown portion, $BEQ_{unknown}$. **(B**) Fractions of the concentrations per chemical C_i for the six chemical categories in 591 plasma extracts. **(C)** REP_i (eq. 3) for the 75 quantified and neurotoxic chemicals compared to narciclasine, divided in the six chemicals categories. **(D)** The tip of the iceberg or BEQ_{chem} is defined as mixture effects based on the concentrations and potencies of identified and quantified chemicals (eq. 4) in the plasma extracts and was divided into the six chemical categories. BEQ_{chem} can be further divided into the contributions of the individual chemical categories: industry and consumer goods, BEQ_{ind}; pesticides, BEQ_{pest}; pharmaceuticals, BEQ_{pharm}; personal care products, BEQ_{pers}; food and other consumption, BEQ_{food}; and endogenous compounds, BEQ_{end}. **(E)** Relative contributions to BEQ_{chem} of the respective chemical groups for the 591 plasma extracts. **(F)** The distributions of active BEQ_{bio} (n = 502), $BEQ_{unknown}$ (n = 439), BEQ_{matrix} (n = 9) and BEQ_{chem} (n = 502). BEQ_{matrix} was derived from the extracted charcoal-stripped pooled plasma samples. **(G)** Portion of BEQ_{bio} explained by BEQ_{chem}. BEQ_{bio} were matrix-corrected by subtracting the median BEQ_{matrix} of 14.7 $ng_{narciclasine} \cdot mL_{plasma}^{-1}$.

Fig. 3. Confirmation of additive effects of complex mixtures.
(A) The mixture effects of the identified chemicals (concentrations in table S3, relative effect potencies in table S13) were extrapolated to a relative enrichment factor (REF) of 1, which equals that of undiluted plasma (table S17). The effects of all chemicals or individual categories were calculated from the concentration-effect curves of the single chemicals with the model of concentration addition (eq. 4). **(B)** BEQ_{chem} and respective BEQ_i of the 75 tip-of-the-iceberg chemicals for the 591 plasma extracts (table S16). **(C)** Distribution of the relative effect potencies (REP_i) as a function of hydrophobicity expressed by the liposome-water distribution ratio $D_{lip/w}$ (table S13) for 61 chemicals in the designed mixtures with dimethyl sulfoxide (DMSO) background (table S25). **(D)** Concentration fractions of the 61 chemicals used for the 63 designed mixtures with DMSO background containing 4 to 28 chemicals per mixture (table S25). **(E)** Effect fractions of the 61 chemicals mixed as described in (D) (table S26). **(F)** Comparison of the measured bioanalytical equivalent concentrations ($BEQ_{bio,mix}$, in units of $ng_{narciclasine} \cdot mL_{plasma}^{-1}$) and predicted $BEQ_{chem,mix}$ (eq. 4) for the 63 designed mixtures with DMSO background (table S26). **(G)** Distribution of the REP_i as a function of $D_{lip/w}$ (table S13) for 45 chemicals in the 15 designed solvent-free mixtures (table S27). **(H)** Concentration fractions p_i of the 45 chemicals used for the 15 designed solvent-free mixtures containing 7 to 16 chemicals per mixture (table S27). **(I)** Effect fractions $effect_i$ of the 45 chemicals mixed as described in (H) (table S27). **(J)** Comparison of the measured $BEQ_{bio,mix}$ and predicted $BEQ_{chem,mix}$ for the 15 designed solvent-free mixtures (table S28).

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Supplementary Materials

Materials and Methods

Supplementary Text S1 to S7

Figs. S1 to S11

Tables S29 to S33

References (*93*–*108*)