ARSENIC EXPOSURE AND DEVELOPMENTAL NEUROTOXICITY: AN EVALUATION OF BIOMARKERS OF EFFECT

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ABBREVIATIONS

Acetylcholinesterase (AChE)
Adverse outcome (AO)
Adverse outcome pathways (AOPs)
Arsenic (As)
Arsenic pentoxide (As(V))
Mitogen-activated protein kinase (MAPK)
Mono-methylated arsenic (MMA)
Molecular initiating event (MIE)
Nicotinamide-adenine dinucleotide phosphate (NADPH)

Arsenic trioxide (As(III))
As(III)-methyltransferase (As3MT)

Attention-deficit/hyperactivity disorder (ADHD)
Autism spectrum disorders (ASD)
Benchmark dose lower confidence limit (BMDL)
Blood-brain-barrier (BBB)
Body mass index (BMI)
Body weight (bw)
Bone morphogenic protein 2 (BMP2)
Brain-derived neurotrophic factor (BDNF)
Central nervous system (CNS)
Cornu ammonis (CA)
C-reactive protein (CRP)
Ca^{2+} /calmodulin-activated kinase CaMK
cAMP-responsive binding protein (CREB)
Catalase CAT

Central nervous system (CNS)
Centers of Disease Control and Prevention (CDC)
Cerebrospinal fluid (CSF)
Cornu ammonis 1 (CA1)
Cyclic adenosine monophosphate (cAMP)
Cyclooxygenase 2 (COX-2)
Deiodinase (DIO)
Developmental neurotoxicants, (DNTs)
Di-methylated arsenic DMA
Enzyme-linked immunosorbic assays (ELISAs)

European Human Biomonitoring Initiative (HBM4EU)
European food safety authority (EFSA)
Free T3 (fT3)
Free T4 (fT4)
functional magnetic resonance imaging (fMRI)
Gamma amino butyric acid (GABA)
Glucocorticoid receptors (GR)
Gestational day (GD)
Glutamic acid decarboxylase (GAD)
Glutathione (GSH)

Glutathione S-transferase (GST)
Growth hormone (GH)
Guanosine triphosphate (GTP)

Gyrus dentatus (GD)
Hippocampal-pituitary-adrenal axis (HPA-axis)
Indoleamine 2,3- dioxygenase (IDO)
Inorganic arsenic (iAs)
Insulin growth factor-I (IGF-1)
Interferon-γ (IFN-γ)
Interleukin-1β (IL-1β)
Joint FAO/WHO Expert Committee on Food Additives (JECFA)
Key events (KEs)
Key Event Relationship (KER)
Kynurenine (KYN)
Lipopolysaccharide (LPS)
Long-term depression (LTD)
Long-term memory (LTM)
Long-term potentiation (LTP)
Lower bound (LB)
Major depressive disorder (MDD)
Mature BDNF mBDNF

Nanogram (ng)
Na+/I- symporter (NIS)
Neurodevelopmental disorders (NDDs)
N-methyl-D-aspartate receptor (NMDAR)
n-myc downstream-regulated gene 4 (NDRG-4)

Organisation for Economic Co-operation and Development (OECD)
Peroxisome proliferator-activated receptor gamma (PPARγ)
Phosphoinositide 3-kinase (PI3K)

Phospholipase Cγ1 (PLCγ1)

Polybrominated diphenyl ethers (PBDEs)
Provisional Tolerable Weekly Intake (PTWI)
Quantile-based g-computation (QGC)
Reactive oxygen species (ROS)
S-adenosylmethionine (SAM)

Sirtuin-1 (SIRT-1)
Small mothers against decapentaplegic 1/5 (SMAD 1/5)
Socio-economic status (SES)
Sulfhydryl (SH)
Superoxide dismutase (SOD)

Thyroid hormone (TH)
Thyroid hormone-responsive element (TRE)
Thyroperoxidase antibodies (TPOAB)
Thyroxine (T4)
Thyroxine binding globulin (TGB)

Tyrosine kinase receptor B TrkB

Thyrotropin-releasing hormone (TRH)
Thyroid stimulating hormone (TSH)
Tissue plasminogen activator (tPA)

Total arsenic content (tAs)
Triiodothyronine (T3)
Total T3 (TT3)
Total T4 (TT4)
Transthyretin (TTR)

Tryptophan (TRP)
Tryptophan dioxygenase (TDO)
Tumor necrosis factor-α (TNF-α)
Tpper bound (UB)
World Health Organization (WHO)
5'-C-phosphate-G-3' (CpG)

ABSTRACT

Arsenic (As) is an ubiquitous element in the human environment, and exposure to As poses a significant threat to humans of all age groups. Humans are exposed to As through ingestion of foodstuffs and drinking water as well as by inhalation. The prenatal period, in addition to the postnatal developmental period of children and adolescents, are life phases where they are particularly at risk for potential damages to the central nervous system (CNS) due to arsenic exposure. These damaging effects may be too subtle to be detectable with measurement methods currently available and might only become manifest in adulthood. Therefore, it is vitally important to find detection tools that are sufficiently sensitive. One such potential detection tool might be effect biomarkers, that may be used in the monitoring of adverse effects to the CNS, and that may be used as early warning systems before irreversible damages occur. In this master's thesis, an attempt was made to identify such effect biomarkers. This was done by conducting a comprehensive literature search after human, animal and *in vitro* studies published during the last 10 years that had examined neurotoxic effects of arsenic exposure. In order to facilitate the identification of effect biomarkers, the AOP Wiki, with adverse outcome pathways (AOPs) relevant for developmental neurotoxicity, was used. As a result, two effect biomarkers; brain-derived neurotrophic factor (BDNF) and thyroid hormones (THs), were selected, and a total of 31 human, animal and *in vitro* studies were selected and identified, where these effect biomarkers had been used. In the evaluation of their usability in future, human studies, toxicity mechanisms suggested in these studies were investigated, and the AOP Wiki was used to find consistencies between main findings in these studies and AOPs relevant for developmental neurotoxicity. In addition, AOP wiki was used to identify toxicity mechanisms that may affect the levels of BDNF and THs that were not suggested in the literature identified during the literature search. Furthermore, in the evaluation of these effect biomarkers, limitations of the human, animal and in *vitro* studies that were selected and identified during the literature search were investigated. In addition, in this thesis, it was focused on various considerations regarding the measurement of these biomarkers in

humans. Only one human study, focusing on associations between arsenic exposure, BDNF and neurobehavioural endpoints, respectively, was identified. Furthermore, in the selected and identified animal studies that used BDNF as a biomarker, several consistencies between main findings and relevant AOPs were found, which strengthens the relevance of BDNF as an effect biomarker in epidemiological studies. In the literature it was, amongst others, suggested that arsenic's effects on BDNF was mediated by oxidative stress. Furthermore, involvement of phosphorylation of cAMP-responsive binding protein (CREB) was suggested as a mechanism. Other studies suggested that arsenic's effects on BDNF was mediated by disruption of serotonergic signalling, BDNF DNA methylation and histone methylation. In addition, in one study, it was suggested that arsenic's effect on BDNF was mediated by increased bone morphogenic protein 2/ small mothers against decapentaplegic 1/5 (BMP2/Smad 1/5) signalling. Furthermore, in other studies, associations between arsenic exposure, BDNF and learning and memory impairments were reported. These adverse neurobehavioural effects were, in turn, shown to be associated with for example altered hippocampal and dendritic morphology. Likewise, for TH, several consistencies between AOPs relevant for developmental neurotoxicity and main findings in the selected and identified animal studies were found. In the experimental studies, it was, for example, reported reduced expression of Ca^{2+} /calmodulin-activated kinase IV (CaMKIV) due to arsenic exposure, learning and memory impairments and neurodegenerative changes in the hippocampus, cerebrum and cerebellum.

One of the main strengths of BDNF as a potential effect biomarker in future, human studies of arsenic exposure is that it has previously been suggested as an effect biomarkers in human biomonitoring studies examining effects of exposure to other environmental toxicants on BDNF. One of the main limitations of its future use, however, is the small number of human studies of arsenic exposure, where BDNF has been implemented as a biomarker. Conversely, a comparably large number of human studies exist, where negative associations between arsenic exposure and THs have been found. However, as opposed to BDNF, which has been used as an effect biomarker in several experimental studies, comparably few animal and *in vitro* studies have investigated associations between arsenic exposure, effects on THs and neurodegenerative or neurobehavioural effects. Weaknesses identified in human studies of associations between arsenic exposure and TH dysregulation

included inconsistent adjustment for covariates and that procedures for measurements of TH were not uniform and standardized. In addition, different TH parameters were used. Furthermore, a large number of the human studies were cross-sectional, which precludes the possibility of linking causes to effects. Both BDNF and TH are together included in an AOP describing developmental neurotoxicity, and it is likely that an increased sensitivity and specificity can be achieved if they are being used in combination as effect biomarkers.

1.0 INTRODUCTION

Arsenic (As) has been called the king of poisons and the poison of kings and, more than any other, this infamous and ubiquitous element has influenced the life of human populations for thousands of years. Presently, chronic exposure to arsenic, from air, water, food and soil, causes a high prevalence for adverse health effects, both in developed and developing countries(1). Our knowledge of such effects, as well as our understanding of the global magnitude of the problems associated with arsenic exposure, continues to grow(1).

As a consequence of industrial pollution, close to all human beings are currently at risk of being chronically exposed to lower or higher levels of toxic metals. Among these, lead, cadmium and arsenic, are particularly hazardous, and arsenic is both more toxic and abundant than both lead and cadmium(2). As of 2014, 214 chemicals, among them, arsenic and arsenic compounds, were identified as neurotoxicans(3). Partly, because our understanding of the routes of exposure is limited and, partly, because it is virtually impossible to remove them from the environment, heavy metals make humans permanently at risk. According to Dani (2010), arsenic is particularly hazardous because of 1) negative effects on growth and development in a large group of humans, animals, plants and microbial species, 2) bio-availability, 3) environmental availability, 4) broad geographical distribution, transportability and persistence in ecological niches, 5) chronic toxicity and tissue accumulation, 6) invisibility as well as taste- and odourlessness when dissolved in water and 7) toxic potential(4). The toxicity and bioavailability of inorganic arsenic (iAs) depends on its valence state, and trivalent arsenic trioxide (As(III)) has been shown to be more harmful to animals than pentavalent arsenic pentoxide (As(V))(5).

Groundwater is a source of drinking water for large parts of the global population, and it is also an important source of arsenic exposure(6). Because of this, the World Health

Organization (WHO) has developed guidelines for maximum permissible levels of arsenic in groundwater. The growing awareness and knowledge of adverse effects of exposure is reflected in a reduction of these guideline levels from 50 to 10 µg/L(7).
Globally, it is estimated that more than 230 million people are exposed to arsenic at concentrations exceeding these guideline levels, due to ingestion of arsenic-contaminated drinking water(8).
Arsenic's carcinogenic effects and systemic toxicity have been thoroughly described, and it is for example known to have toxic effects on the hepatic, respiratory, gastrointestinal and haematopoietic organ systems. In fact, arsenic is known to affect almost all of the body's tissues and organs. Comparably less is known about how arsenic affects the developing central nervous system (CNS)(1). The developing human brain is exceptionally sensitive to disturbances in its homeostasis, e.g. by toxic insults. Chemicals interfering with brain developmental processes (developmental neurotoxicants, (DNTs)) can lead to irreversible neurodevelopmental deficits, behavioural problems and related disorders even at exposure levels that may be considered "safe" for adults(9). Here, the use of inverse commas is justified due to knowledge gaps, which makes it unclear, which levels of arsenic exposure might, indeed, be considered safe. Adverse effects of pre-, postnatal or adolescent exposure to arsenic or other DNTs might reveal themselves during childhood or adolescence. Alternatively, adverse effects of exposure might only become manifest during adulthood(10).
The incidence of diagnosed neurodevelopmental disorders (NDDs) such as attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) have increased rapidly in developed countries since the 1980s, and this increase has led to concerns that environmental toxicants may be among the contributing factors(11). This increased incidence may be illustrated by statistics from a US national 2016 parent survey, showing that the incidence of ADHD for US children was 6.1 million (9.4%) this year(12). Furthermore, according to statistics from the Centers of Disease Control and Prevention (CDC), the US incidence of ADHD increased by 20% between 2003 and 2007 (this corresponds to an annual increase of approximately 5.5%). As a contrast, between 1997 and 2006, the annual increase was approximately 3%(13). Most probably, these changes have occurred independently of any major changes in diagnostics methods(11). Regarding ASD, there has also been a dramatic increase in diagnoses in developed countries: Whereas 1 in

5.000 Americans were diagnosed with the disorder in 1975, 1 in 88 were affected in 2009, in some areas(14). Less than 40 % of this increase is thought to be explainable due to changes in diagnostic tools or due to greater social awareness. Furthermore, since the gene pool has remained unchanged, this means that environmental factors, somehow must be implicated(11).

Both pre- and postnatal arsenic exposure poses a threat to human health: iAs is considered more toxic than methylated and other organic arsenic species, and both iAs and its metabolites appear to cross the placental barrier virtually unhindered(15–18). Furthermore, by accumulating in the placenta, arsenic may impair placental blood flow and disturb placental vasculogenesis, which, in turn, might impair fetal growth(19–21). Added to this, comes the fact that arsenic may, just as easily as it crosses the placental barrier, cross the blood-brain-barrier (BBB): It has, for example, been shown to accumulate in brain structures like the cortex, hippocampus and striatum(22,23). Another factor, which makes the foetus vulnerable, is its still immature kidneys, with limited ability to eliminate toxicants(1).

1.1 EVIDENCE FROM EPIDEMIOLOGICAL STUDIES

Of the numerous studies that have reported associations between arsenic exposure and negative effects on cognitive function in children, the majority have been cross-sectional(24). Thus, based on the majority of them, it is not possible to link arsenic exposure as a cause, to cognitive impairments as an effect. Epidemiological studies, in particular, those conducted on children in developing countries, have shown associations between for example reduced IQ and other cognitive impairments and arsenic exposure, respectively(25). Memory impairment and reduced IQ have, for example, been shown among Taiwanese(26), Bangladeshi(27–29), Mexican(30–33) and Indian children(34). Furthermore, among the IQ subcategories, arsenic seems to affect verbal IQ most severely in children(24). These adverse effects can also be seen in adolescents, which suggests that they prevail, and that effects likely occur as a consequence of cumulative exposure, rather than as a result of isolated incidences of acute exposure. Furthermore, although few studies yet have addressed it, it appears that females are at greater risk than males (24). Although such effects have been seen at high exposure levels, similar effects have also been reported at comparably low levels of chronic exposure (<10 μg/L)(35). However, despite a large body

of evidence from epidemiological studies indicating adverse effects on IQ and other aspects of cognitive functioning, no irrefutable associations have yet been found(25). This is, in part, due to the already mentioned fact that the majority of epidemiological evidence stems from cross-sectional studies(24) and partly because the majority of these studies have been conducted in developing countries, where factors like co-exposure to other toxicants, malnutrition, co-morbidities and low socio-economic status (SES) may act as confounders(25). In addition, co-exposure to other toxicants likely complicates the ability to draw firm conclusions. This is because other metals like Hg, Mn, Cd and Pb might co-occur with As in the environment. Furthermore, in their environment, humans are exposed to a large number of other chemicals. Together, this mixture is a "toxic cocktail", which may share some of arsenic's toxicity mechanisms. Among these compounds are for example bisphenols, phthalates and polybrominated diphenyl ethers (PBDEs)(11). Together with arsenic, these other metals and chemicals may have synergistic effects, which can make it difficult to determine the isolated effect of arsenic, and few epidemiological studies have addressed this issue(24). Furthermore, low SES makes it difficult to draw firm conclusions: Low SES due to poverty is associated with increased severity of perceived stress, and low SES has, in turn, been shown to be significantly associated with neurodevelopmental disorders(36). In addition, low SES frequently co-occurs with less education, and parental education level has been shown to correlate with cognitive function in children exposed to arsenic(34).

Malnutrition is another factor that cannot be ignored: Indeed, even in cases where effects of exposure to arsenic or other heavy metals and chemicals can be ruled out, malnutrition can severely affect cognitive development. Iron deficiency, for example, is known to be correlated with impaired cognition and neurological function(34). This effect may, in turn, be exacerbated by arsenic exposure, since it is believed to heighten the risk of anaemia(38).

Oxidative stress, inflammation, mitochondrial dysfunction and, with that, uncoupling of oxidative phosphorylation, are among the most thoroughly investigated mechanisms by which arsenic exerts its neurotoxic effects(24,39). In this context, selenium deficiency may exacerbate the effects of arsenic poisoning. This is because selenium is a dietary essential trace element with both important antioxidant and anti-inflammatory properties(40,41).

Another important antioxidant, that might be lacking in cases of malnutrition is ascorbic acid, which protects cell membranes from the damaging effects of free radicals(42).
In the body, arsenic is detoxified by methylation. The body's methylation capability is, in turn, thought to depend on vitamin B-12, folic acid, choline, betaine and methionine. These nutrients may be insufficiently supplied through the diet, which, yet again, illustrates that malnutrition may exacerbate the effects of arsenic neurotoxicity(43).

1.2 EVIDENCE FROM EXPERIMENTAL STUDIES

Findings of adverse effects on cognitive function from epidemiological studies are supported by findings from animal studies(24). Here, also, adverse effects on learning and behaviour has been reported(24,103–105). In particular, cognitive functions involving the hippocampus seem to be affected(24). Chronic and acute arsenic exposure has, for example, in rodents been shown to impair short-term memory, working memory and spatial memory(46,48–50). Some of this evidence has been found, using very high exposure levels (many times higher than the WHO guideline values)(24). However, exposure during the perinatal period (prenatal and newborn period) to environmentally representative concentrations (50 µg/L) has for example in rodents been reported to be associated with depressive-like behaviour(45). Similarly, animal studies have reported significant associations between developmental exposure to arsenic in the 50µg/L range and cognitive impairments as well as altered stress responses and adverse effects on neurogenesis(24,45,51). Neurotoxic effects of arsenic may be mediated by genotoxicity (caused, for example, by impaired DNA repair), disrupted cell proliferation and signal transduction, as well as by inhibition of thiol-containing enzymes(52,53). Furthermore, studies also indicate that As may exert neurotoxic effects through for example neuronal apoptosis(54) and impairment of neurite outgrowth(55–57). In addition, arsenic exposure has been reported to be associated with dose-dependent reductions in the expression of N-methyl-D-aspartate receptor (NMDAR) subunits(48,58,59). NMDARs in the hippocampus are believed to play an important role for learning, memory, synaptic plasticity and excitatory synaptic transmission(60). In addition, arsenic has been shown to affect the firing activity of hippocampal neurons and to inhibit long-term potentiation (LTP)(61). The latter is a process, which depends on synaptic plasticity and is thought to be essential for memory formation(62). In addition, morphological changes to hippocampal neurons as a result of

arsenic exposure have been reported(48,49). Furthermore, arsenic exposure has been shown to affect the level of several neurotransmitters. Examples of these include noradrenaline, serotonin and dopamine(24,63–65). Another study, with rats exposed to arsenic, reported reduced gamma amino butyric acid (GABA) levels and reduced glutamic acid decarboxylase (GAD) activity in hypothalamus, cerebellum and brainstem(110). Increased levels of plasma corticosterone (the animal analogue to human cortisol) has also been shown in mice exposed to arsenic, which was correlated with signs of depression(45). In another study, decreased levels of hippocampal glucocorticoid receptors (GR) was reported following arsenic exposure in mice(46). This is relevant, due to the role GRs play in regulating the hippocampal-pituitary-adrenal axis (HPA-axis), which for example is implicated in mood disorders(67). In addition, arsenic has been shown to decrease brain activity of the acetylcholinesterase (AChE) enzyme, which regulates cholinergic neurotransmission (24,68). Furthermore, arsenic is known to affect levels of brain-derived neurotrophic factor (BDNF)(69) and thyroid hormone (TH)(70), and later in this thesis, evidence from human and animal studies of arsenic's effects on BDNF and THs will be presented.

1.3 SOURCES OF ARSENIC EXPOSURE

Drinking water and metal dust particles are the main sources of chronic exposure to iAs(71,72). Important sources of anthropogenic exposure are pesticides and herbicides(21) as well as pollution related to coal, hard rock mining and petroleum industry(4). In addition, iAs is used in semiconductors and as a chemotherapeutic agent(73). To describe arsenic's toxicity mechanisms is challenging for several reasons: Partly, this is because there is a large number of arsenic species. Yet another, is the fact that various arsenic species may have different toxicities that, in turn, depend on their oxidation state. Of these, trivalent, inorganic arsenic (iAs(III) compounds are, as mentioned, thought to be the most toxic(1).

1.3.1 Arsenic in groundwater

Groundwater contamination with arsenic is mostly geogenic(1). In Bangladesh, it is calculated that around 50 million people are at risk of exposure through groundwater(1,74,75). In addition to Bangladesh, other severely affected countries are India(76–80), China(81), Vietnam(82), the USA(83), Indonesia(68), Laos(85), Myanmar(70) and Nepal(87). Furthermore, populations in countries like for example South

Africa, Mexico, Pakistan, Canada, Hungary, Chile and Argentina are also believed to be at risk(88).

1.3.2 Arsenic in soil

Uncontaminated soils are thought to have arsenic concentrations ranging from 1-40 mg/kg, and the lowest concentrations are found in soils derived from granites, as well as in soils that are sandy. Larger concentrations are, on the other hand, found in organic and alluvial soils(89). Furthermore, soils near smelters or mining areas, as well as soils treated with arsenic containing pesticides, may have high concentrations(6).

1.3.3 Arsenic in air

Arsenic is usually present in ambient air as a mixture of arsenate and arsenite(90). In the United States, mean values of arsenic in air have been shown to be between <1 to 3 nanograms (ng)/m³ in rural regions and in the range of 20-30 ng/m³ in urban ones(59). Mean air concentrations in England have been reported to be 5.4 ng/m³ (92). Furthermore, in some cities, air concentrations may be several hundreds of ng/m³, and in the vicinity of non-ferrous metal smelters, air concentrations may lie above 1000 ng/m³ (93).

1.3.4 Dietary sources of arsenic exposure

Currently, no limit for maximum dietary iAs exposure has been established (94). Earlier, a provisional tolerable weekly intake (PTWI) of iAs was agreed upon, which subsequently was withdrawn by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2011. Instead, a benchmark dose lower confidence limit ($BMDL_{05}$) was established, at 3 µg/kg body weight (bw) per day(95). In 2021, the European food safety authority (EFSA) published a report on chronic dietary exposure to iAs in the European population(96). In toddlers, the estimated daily highest mean dietary exposure was 0.30 µg/kg bw at the lower bound (LB) and for both infants and toddlers, the daily highest mean dietary exposure was 0.61 µg/kg bw at the upper bound (UB). Furthermore, the highest daily exposure at the 95th percentile was estimated to be 0.58 µg/kg bw for toddlers and 1.20 µg/kg/bw for infants(96). In 2009, the EFSA Panel on Contaminants in the Food Chain established a benchmark dose lower confidence limit ($BMDL_{01}$) at 0.3-8 µg/kg bw per day for a 1% increase in risk of lung, bladder and skin cancers as well as for a 1% increased risk of skin lesions(97). Generally, the estimated levels of mean LB dietary exposure were in the EFSA

report from 2021 reported to be below these values. However, in the case of the LB dietary exposure levels in the 95 percentile range, the estimated maximum exposures for infants, toddlers and older children were within the $BMDL_{01}$ range at 0.3-8 µg/kg bw per day. For all age groups, main contributors to dietary iAs exposure at the LB were drinking water, "grains and grain-based products (no rice)" and rice. Main uncertainties concerning the estimations of dietary iAs exposure were related to methodology as well as related to the fact that information about consumption of certain foodstuffs with iAs-containing ingredients as well as information about the occurrence of these ingredients in foodstuffs, was lacking. In addition, another main uncertainty concerning the estimations was how food preparation affects iAs levels(96). Young children belong to the group most heavily exposed to iAs. Partly, this is due to the dietary habits of young children, for example illustrated by the fact that infants and toddlers consume a higher amount of rice-based products. Furthermore, young children consume higher amounts of food in relation to body weight compared to older humans(98).

In several countries around the world, seafood is recognized as the main source of human As exposure, and concentrations of As in many types of fish and shellfish exceed to a large degree As concentrations found in terrestrial foods(99). It is important to notice, however, that organic, and not inorganic As species, dominate in marine foods, and that organic arsenic species generally are considered to be less toxic than inorganic ones. In most seafoods, iAs concentrations are negligible(99). One notable exception, however, is the brown algae hijiiki, which contains high amounts of various arsenic species, of which the main fraction is iAs. It is also known that other species of brown algae can contain high amounts of iAs(100). Furthermore, crustaceans, particularly molluscs, can contain significant amounts of iAs (mean of published data: 130 ng/g). Levels in edible seaweeds can be several thousand ng/g, particularly in species like hijiiki(97). Low concentrations of mono-methylated and di-methylated arsenic species (MMA and DMA respectively) can be found in marine foods. Furthermore, methylated arsenic species found mainly in marine foods are for example trimethylarsine oxide (TMAO), arsenocholine (Asc), arsenobetaine (AsB) and tetraarsenic oxide (TETRA)(101). There is limited available information about the toxicity of organoarsenicals and effects of long term exposure¸which makes it uncertain to what extent ingestion of seafoods poses a threat to humans. Too few data still exist on distributions and concentrations of organic arsenic species in seafood(101). Added to this

comes the fact that only a few human population studies have been conducted, and that very few toxicity data exist. It is therefore currently not feasible to assess risks associated with exposure to organoarsenicals. Several organoarsenicals have still not been tested for toxicity(101).

1.4 ABSORPTION, TISSUE DISTRIBUTION, METABOLISM AND EXCRETION

1.4.1 Absorption and tissue distribution (iAs)

There are several factors that influence distribution and retention of As in the body. Among these are for example route of exposure, arsenic species, genetic factors, methylation capacity and dose level(6). Arsenite (As 3+) as well as arsenate (As 5+) bind with free sulfhydryl (SH) groups. However, the binding affinity of As 3+ to these groups is stronger than that of As 5+. As a consequence, retention of As 3+ is substantially higher than retention of As 5+(102). In humans, iAs has a half-life of ca. 10 hours(6). Furthermore, the retention time of iAs varies between organs, and the longest retention time for iAs in mammalian tissues, has in experimental studies been observed in the upper gastrointestinal tract, epididymis, skin and hair(103). For trivalent iAs species, 95% of the administered dose has been shown to be absorbed in the gastrointestinal tract(6). In addition, animal studies have shown iAs to accumulate in brain tissue(17,104). For example, after daily exposure to sodium arsenite (2.5, 5 and 10 mg/kg/day), arsenic metabolites were found in medulla oblongata, pons, pituitary gland, striatum, midbrain, hippocampus, cerebral cortex and striatum of mice(105). Whereas the transfer from mother to child of As via the placenta appears to be extensive(15–18), very little As seems to be excreted in breast milk(6). Researchers examining Argentinian children, for example, reported that urinary arsenic concentrations of infants decreased from ca 80 µg/L two days after birth to below 30 µg/L at 4 months of age(15). Concentrations of arsenic in maternal blood and urine were on average 10 and 320 µg/L, respectively, whereas mean arsenic level in breast milk was 2.3 µg/L- in other words, much lower(15). These findings are supported by a study on Bangladeshi infants, that found very low concentrations of As in breast milk, despite high concentrations in drinking water. This suggests that maternal methylation protects small children from exposure(106).

1.4.2 Metabolism of iAs

In the human body, metabolism is a detoxification process, by which iAs is converted to methylated species that subsequently are excreted. For this process, the Challenger pathway has been suggested as a mechanism(6). This pathway involves a series of reduction and oxidation steps, that convert iAs from pentavalent As (V) to As (III). The reduced, trivalent As(III) (arsenite) is both highly reactive and toxic, and, among the end products, MMA(V) and DMA(V) are formed(6). Furthermore, DMA(III) and MMA(III) are also created, and both of these molecules have also been shown to be highly toxic and reactive(79). When humans are exposed to arsenates or arsenites, this results in increased levels of urinary DMA (DMA (III) and DMA (V)), MMA(V), As(V) and As(III)(80,81). After uptake, iAs(V) is reduced to iAs(III), which subsequently is conjugated with glutathione (GSH)(53). In addition to GSH conjugation, As(III) is methylated by As(III)-methyltransferase (As3MT), and as end products, monomethylated (MMA(III or V)) and dimethylated (DMA (III or V) metabolites are created(110). In the conversion, reduction and detoxification of arsenate to arsenite, GSH can function as a donor of methyl groups(111). It can therefore mediate detoxification, and because the brain uses GSH in detoxification processes, arsenic may reduce the brain's supply of GSH(112). This is because permeability of the BBB for GSH is low, and its availability within the CNS is dependent on *de novo* synthesis(113). The liver appears to be the main site of methylation, and methylation is provided by methyl-transferases, using S-adenosylmethionine (SAM) as co-substrate(114,115). Furthermore, it has been suggested that the gut microbiota may play a role in the pre-systemic metabolism of arsenicals: Enzyme systems located in the gastrointestinal flora may interact with other metabolizing enzymes(116).

1.4.3 Excretion of iAs

The major part of the excretion after exposure to iAs takes place via the kidneys and urinary excretion rate depends on arsenic compound(6). Furthermore, at low exposure levels, there has been shown a linear relationship between levels of urinary As and levels of As intake(117). In addition, humans with a continuous intake of As over a few days, have been reported to excrete 60-70% of daily intake via urine(118).

1.5 BIOMARKERS OF NEUROTOXICITY

This master's thesis will focus on effect biomarkers. Briefly, effect biomarkers can be structural, behavioural, physiological, biochemical, cellular or molecular, and enable the measurement of changes in an organism as a result of for example exposure to environmental toxicants(10). In this context, one main aim of this thesis is to present and evaluate effect biomarkers of neurotoxicity that, until now, mainly have been restricted to animal studies of neurotoxic effects of arsenic exposure and that may be used as effect biomarkers in future human studies. This evaluation will partly be based on existing information about neurotoxicity pathways, suggested in experimental studies.
A myriad mechanisms of action and toxicity pathways have been suggested, by which arsenic may exert its neurotoxic effects(25), and this thesis will focus on some of these. The term "neurotoxicity" can be defined as *"any adverse effect on the chemistry, structure or function of the nervous system, during development or at maturity, induced by chemical or physical influences"(119)*. Morphological changes, such as myelopathy (characterized by loss of glial cells surrounding the axon), neuronopathy (loss of neurons), or axonopathy (degradation of the axon) are considered to be important neurotoxic effects. Other, equally important neurotoxic effects, are those morphological changes that are either mild or partially reversible. Furthermore, neurotoxic and adverse effects can occur, even though no structural damage is involved, and even in cases where the effects are fully reversible(9).

In the definition above, the developmental period is mentioned as a phase, during which humans are particularly susceptible to neurotoxic effects. This is partly due to the BBB, which remains poorly developed during the prenatal period, and therefore does not properly protect the nervous system against toxicants(9). Despite of this, however, the developing nervous system has some advantages over the *developed*: Compared to the developed nervous system, it may for example be more flexible and more able to compensate for functional losses caused by exposure to toxicants. However, if these damages occur during key periods of brain development, they may be irreversible(120). In addition, neurodegenerative effects might arise due exposure to very low concentrations of a chemical, if this exposure takes place during an extended period of time (120). Broadly speaking, biomarkers fall into three categories: 1) exposure biomarkers, 2) biomarkers of susceptibility and 3), effect biomarkers(39).

1.5.1 Biomarkers of exposure and susceptibility

During human bio-monitoring, the concentrations of environmental chemicals or their metabolites in body fluids or tissues (for example nails, hair, blood or urine) are measured using biomarkers of the first category, namely biomarkers of ***exposure***. In addition, soil and water samples can be used as biomarkers of exposure, to measure concentrations of a toxicant. These biomarkers provide a measure of the actual exposure to specific environmental chemicals and facilitate the evaluation of relationships between exposure and adverse health effects, to support regulators or policy making(121).

Biomarkers of susceptibility are indicators of factors determining individual vulnerability to adverse effects of a toxicant and they are helpful in defining developmental time periods, where susceptibility is particularly high. Susceptibility biomarkers may for example be genetic polymorphisms or chromosomal aberrations(121). In the case of arsenic exposure, polymorphisms can for example exist in the AS3MT gene, which encodes an enzyme involved in the methylation and detoxification of arsenic. Such polymorphisms may render an individual more susceptible to adverse effects(122–124). Furthermore, these polymorphisms can exist alone or simultaneously with polymorphisms in the gene for e.g. glutathione S-transferase (GST), and may contribute to individual differences in susceptibility to negative effects of arsenic exposure(124). This is because GST plays an important role in the detoxification of xenobiotics(125).

1.5.1 Effect biomarkers: Challenges and requirements

Effect biomarkers can, as already mentioned, be structural, behavioural, physiological, biochemical, cellular or molecular, and enable the measurement of changes in an organism as a result of for example exposure to environmental toxicants(9). Furthermore, effect biomarkers are indicators that link exposure to a disease or to a possible or an already established health impairment. As such, their function consists in strengthening the assumed causal relationship between exposure and specific health outcomes(9). The identification of suitable effect biomarkers is a process that draws on knowledge from epidemiological studies, as well as on knowledge about a toxicant’s suggested mechanisms of action, which, in turn, is based on experimental studies(9). Ideally, effect biomarkers should be indicators of changes to the nervous system that precede structural or functional damage. In this sense, they can be thought of as early warning systems. In the process of finding such indicators,

knowledge about the mechanisms of action of neurotoxicants is extremely useful. This information is limited, which is a major obstacle in the search for biomarkers of neurotoxic effects(9). The limited knowledge is, in turn, partly due to the complexity of the nervous system and the large number of a neurotoxicant's cellular and biochemical targets(9). Furthermore, this knowledge is limited, because many manifestations of neurotoxicity are either diverse or unknown. This makes the validation of biomarkers of neurotoxicity much more difficult than the validation of for example biomarkers of hepatotoxicity(9). Apart from being early indicators of potential, future damage, biomarkers of neurotoxic effects should ideally be common, but at the same time specific for all forms of neurotoxicity, caused by different substances and agents. This universality of biomarkers might be beneficial in situations where the mechanisms of action of a toxicant is unknown(9). Furthermore, this universality might be essential in real-world scenarios, where humans are exposed to a large number of environmental toxicants simultaneously(11). In a real-world scenario, this co-exposure and sharing of toxicity mechanisms might, in many cases, imply that neurotoxic effects attributable to arsenic exposure, simultaneously are attributable to exposure to lead, cadmium, manganese or several other environmental toxicants: In some cases, these other elements or toxicants might act in synergy or add to arsenic's neurotoxic effects. In other cases, they might be antagonists(12). Often, experimental animal studies have not accounted for these human, real-world scenarios of arsenic exposure. Indeed, many animal studies have focused on neurotoxic effects, solely of arsenic exposure, instead of investigating neurotoxic effects of *co-exposure* to other elements or compounds, that may frequently co-occur with arsenic in the environment(126). This is one of the challenges associated with identifying potential human effect biomarkers using experimental animal studies. Furthermore, this single-chemical approach is reflected by current, regulatory bodies risk assessments, whose risk and exposure-level evaluations, in many cases, are on a chemical-by-chemical basis(44). Another challenge associated with identifying potential human biomarkers using experimental animal studies, are the existing species differences(127).

Neurological disorders associated with chronic exposure to toxicants can manifest themselves clinically decades after its onset: Adverse effects of exposure that for example occurred prenatally or during early childhood, might not reveal themselves before adulthood(9). Prior to these manifestations, effects of exposure might be subtle and

unidentifiable as a specific disease. This lack of detectability is partly due to the limited sensitivity of the commonly used neurobehavioral biomarkers of effect, and it is therefore an important goal to find biomarkers that enable early detection: Ideally, a biomarker should be able to detect changes in an organism that are both reversible and subclinical. Such early detection, in turn, might enable early intervention(120). In addition, biomarkers of neurotoxicity should ideally enable regular monitoring(121). In most cases, however, the ideal of biomarkers as early warning systems is hard to realize: This is because disruptions to the CNS caused by exposure to environmental toxicants, usually are assessed by epidemiological studies at a time point where neurotoxic insults are well underway and irreversible(9).

In the assessment of neurotoxic changes, for example, specific proteins in body fluids or structural changes, visualized using imaging techniques, can be used as non-invasive or minimally invasive biomarkers in longitudinal assessments(39). Fluid-based biomarkers can be detected in whole blood, serum, plasma, urine and cerebrospinal fluid (CSF). Biomarkers found in CSF can be of particular value, since the CSF circulates in a relatively closed system, meaning that biomarkers indicating effects in other tissues have limited access to it(9). One obvious disadvantage, however, with CSF sampling, is that it is a cumbersome and invasive procedure.

Several neurotoxicants are known to affect neurotransmission and neurotransmitters, and may affect their synthesis, degradation or uptake. Many potential biomarkers exist for the monitoring of these effects. In these, as well as in all other cases, where neurotoxic effects are measured, an important criteria biomarkers of effect have to meet, is that changes that occur in the CNS should ideally be measurable in *peripheral* tissue and be representative of changes taking place in the target tissue(121). Furthermore, biomarkers should ideally have a high degree of sensitivity and specificity(127). High specificity means that a biomarker makes it possible to discern effects on for example the CNS caused by exposure to environmental toxicants from diseases or conditions that are unrelated, with a high degree of accuracy(127). If a combination of several different biomarkers are used, this might provide an even stronger specificity than in cases where just one or two are used(127).

To strengthen the specificity, fluid-based biomarkers may, for example, be combined with imaging biomarkers. Biomarkers detectable with imaging techniques such as functional magnetic resonance imaging (fMRI) and micro positron emission tomography (microPET) have several advantages over other neurobehavioural biomarkers of effect. One of these is that both fMRI and MicroPET enable non-invasive measurements of suitable biomarkers, which is an important factor in their evaluation. Another benefit with these techniques is that their application enables longitudinal studies to be performed. In addition, their use enables subjects to function as their own controls, and measurements are easy to quantify(121). A likely disadvantage with both fMRI and MicroPET, however, has to do with a further criteria biomarkers ideally should be able to meet: They should be measurable with inexpensive methods. This is of a particular concern when an epidemiological study shall be conducted with several thousand participants: Such a study might end up being prohibitively expensive, unless biomarkers are used, that are measurable with available, automated and simple laboratory techniques(129).

Converting potential biomarkers into tools that can be used either clinically or pre-clinically, is a major challenge: In most cases, it is not possible to prove that potential biomarkers have predictive clinical value(127). This is for example illustrated by a paper by Poste (2011): Here, it is mentioned, that, in 2011, there were more than 150.000 papers that claimed to have identified thousands of biomarkers, of which only ca 100, subsequently ended up being used clinically for diagnostic purposes(130). Furthermore, in cases where biomarkers are identified, have standardized routines for handling and collection of samples often not been developed. Due to all these challenges associated with identifying biomarkers suitable for human biomonitoring, national and international collaborations that involve modelling, data sharing and data generation are important (127). An example of one such collaboration is the European Human Biomonitoring Initiative (HBM4EU) project, with participants from 30 countries. Involved in the HBM4EU project are the European Commission, the European Chemicals Agency (ECHSA) and EFSA(131). Another example of such a collaboration is the AOP-Wiki, described in the next section.

1.5.2 Evaluation of biomarkers

One important tool for both the identification and evaluation of effect biomarkers, are adverse outcome pathways (AOPs). AOPs are schematic representations, formed based on existing mechanistic knowledge of toxicity pathways(132). AOPs can, for example, describe how signalling pathways are being perturbed due to exposure to chemicals. They describe a chain of causually linked events that lead to adverse human or ecological health effects at different levels of biological organization(132). AOPs describe toxicity pathways that may be shared by several groups of chemicals. The links in the chain of events of the AOPs are formed by so-called "key events" (KEs), that are detectable, biological changes that occur as a result of for example exposure to a chemical (132). Because of the possible sharing of toxicity mechanisms by several groups of chemicals, AOPs can be thought of as generalizations that describe schematically, empirically supported and biologically plausible chains of KEs. These KEs, in turn, can be measured as changes on a molecular, cellular, tissue, organ, individual and population level(132). KEs can for example be changes in levels of specific enzymes or hormones as a response to exposure to a chemical, which can make these specific enzymes or hormones suitable as effect biomarkers. The first link in the chain of events described by AOPs represents the initial chemical interaction and is called a "molecular initiating event" (MIE). Furthermore, the last link in the chain is called the adverse outcome (AO), and the causal connection between two KEs, is called a "key event relationship" (KER)(132).

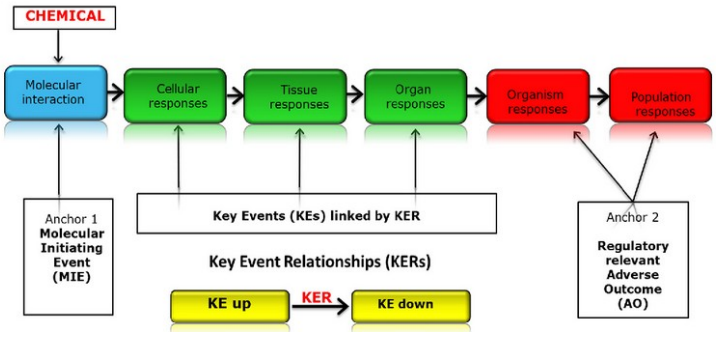

Figure 1. *Schematic representation of an adverse outcome pathway (AOP), taken from Sachana et al (2018) with permission(133).*

In addition, one KE may be shared by multiple AOPs. This illustrates that each individual AOP and its associated KEs, might be elements within a large network of AOPs that, together, provides a more exhaustive description of toxicity pathways and the biological

processes that are implicated(134). AOPs are collected in a publicly accessible database, the AOP-Wiki, available at https://aopwiki.org. AOP-Wiki represents one component of a larger OECD-sponsored AOP knowledge base, and is a joint international effort by researchers. In the assessment of the physiological validity of effect biomarkers, toxicology data about the chemical in question, gathered from experimental studies, together with available AOPs, are used. Together, this provides the scientific justification for using an effect biomarker(135). Reduced levels of, for example, BDNF and thyroid hormone (TH), respectively, are two KEs in several AOPs relevant for developmental neurotoxicity(136–139). Furthermore, BDNF and TH may themselves be usable effect biomarkers in future, human epidemiological studies of exposure to environmental toxicants(140). This illustrates the relevance of AOPs and, with that, KEs as a tool to identify effect biomarkers.

1.6 AIMS OF THE THESIS

The main aim of this thesis was to evaluate the usability of a selection of effect biomarkers of neurodevelopmental toxicity in future human studies of arsenic exposure. This was achieved by:

→ Conducting a comprehensive literature search to identify human, animal and *in vitro* studies of neurodevelopmental effects of arsenic exposure.

→ Identifying KEs in AOPs, relevant for neurodevelopmental toxicity. Some of these KEs may themselves be usable effect biomarkers, and it was focused specifically on studies of neurodevelopmental effects of arsenic exposure where these biomarkers have been used.

→ Describing toxicity mechanisms suggested in selected experimental studies of neurodevelopmental effects of arsenic exposure, in which the selected biomarkers are implicated.

→ Identifying consistencies between main findings in selected studies and KEs in relevant AOPs

→ Discussing strengths and weaknesses with the identified studies

→Exploring other mechanisms, suggested in other studies (not included among the search results), by which arsenic may alter the levels of selected biomarkers and discussing various considerations for their measurement.

2.0 METHODOLOGIES

A comprehensive literature search was conducted in the Pubmed database, with MeSH and non-MeSH search terms listed in **table 1 (See chapter 6** APPENDIX**)** after full-text articles, written in English and published between June the 1st 2012 and June the 1st 2022. Using Boolean operators ('AND' and 'OR'), search terms for health endpoints related to developmental neurotoxicity were combined with search terms for As exposure. This way, a total of 3020 articles were identified. In the next steps, a number of these articles were excluded: Articles not written in English and articles, where the word 'arsenic' was not mentioned in the abstract were ignored. The same was the case for reviews, duplicates of articles and studies that did not include effect biomarkers or relevant health endpoints. Furthermore, non-original research articles were omitted. Following this, it was identified potential effect biomarkers and neurobehavioural endpoints associated with arsenic exposure, by screening of the abstracts of remaining human, animal and *in vitro* studies. In this identification of effect biomarkers, AOPs suggested for neurodevelopmental toxicity was used as a tool: It was sought specifically after effect biomarkers that are KEs in relevant AOPs suggested for neurodevelopmental toxicity. Based on these criteria, two effect biomarkers were selected: BDNF and TH. In the next step, it was searched within the search results after studies where these effect biomarkers had been used. Subsequently, a new literature search was conducted in the Pubmed and Clarivate Analytics Web of Science™ databases, respectively, using the search terms: "Arsenic* AND (Brain-derived neurotrophic factor OR BDNF OR thyroxine OR triiodothyronine OR Thyrotropin OR Iodide Peroxidase OR sodium-iodide symporter)" It was sought after after full-text articles, written in English and published between June the 1st 2012 and June the 1st 2022. This way, a total of 56 (Pubmed search) and 53 articles (Web of science search) were identified, and a number of these were excluded, based on the criteria mentioned above. As a result of the combined literature searches in Pubmed and Web of Science, respectively, 10 articles focusing on effects of arsenic exposure on BDNF (1 human study, 9 animal and *in vitro* studies) and 21 articles focusing on effects of arsenic exposure on TH (13 human and 8 animal studies) were selected and identified. A flow-chart illustrating the selection and identification process is shown in **figure 2**.

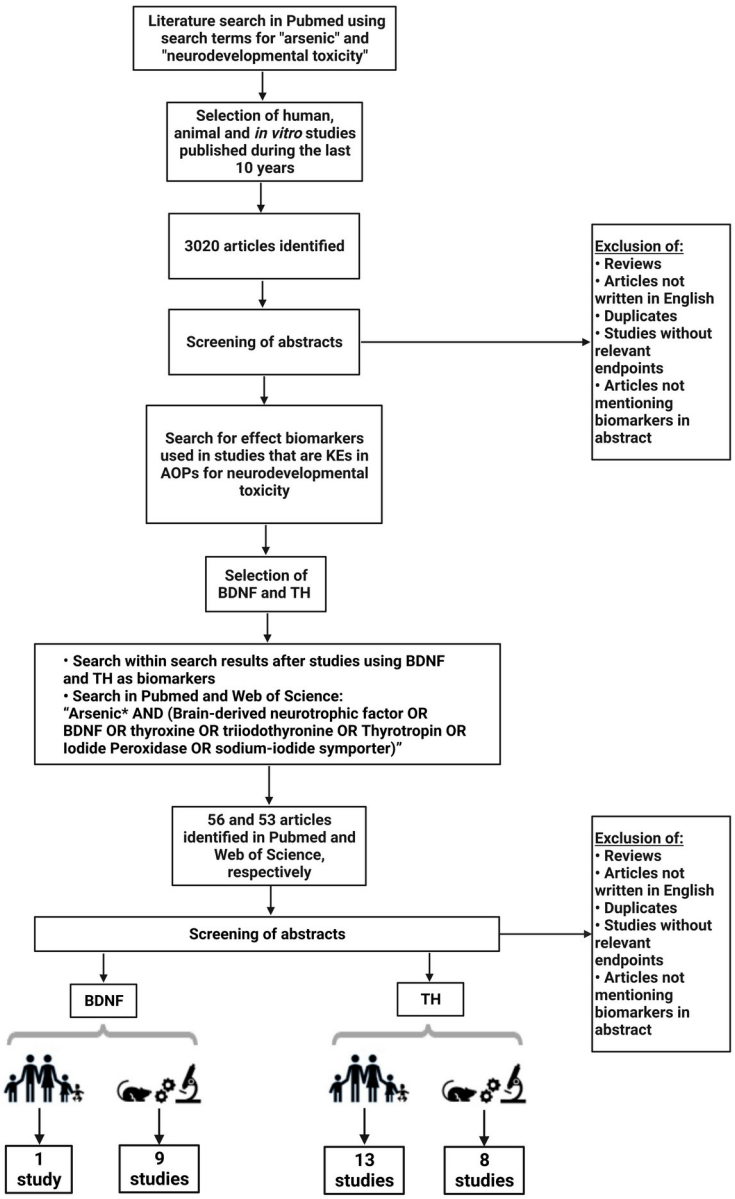

Figure 2. Flow chart showing the process leading to selection and identification of effect biomarkers

3.0 RESULTS AND DISCUSSION

As mentioned, 10 articles focusing on effects of arsenic exposure on BDNF (9 animal and *in vitro* studies (table 3) and 1 human study (**table 4**)) and 21 articles focusing on effects of arsenic exposure on TH (8 animal (**table 5**) and 13 human studies(table 6)) were selected and identified during the literature search. In several of these, a common finding was that arsenic induced oxidative stress(58,141–148). In addition, in some of these studies, it was suggested that the observed neurotoxic effects were, in part, mediated by oxidative stress(58,141,143,144,149). Thus, for the sake of clarification, it will first be described briefly how arsenic species might cause oxidative stress.

Arsenic and oxidative stress

Arsenic may cause generation of reactive oxygen species (ROS) by binding to ligands containing sulphur groups or by its binding to thiol (SH) groups of macromolecules(150). Virtually all As (III) species cause generation of cellular oxidative stress via ROS(151–153). Arsine (AsH_3), along with other redox-active arsenic species, are important for the generation of ROS, by facilitating the production of highly reactive OH• radicals from H_2O_2 via the so-called Fenton reaction(154). Furthermore, arsenic contributes to the production of H_2O_2 via the oxidation of As (V) into As (III)(42). In addition, As is implicated in the formation of IO_2 (singlet oxygen)(154).

It it, currently, still unclear how arsenic causes the generation of oxidative stress(39). It has, however, been suggested that arsenic interferes with complex I and III of the mitochondrial electron transport chain, and that generation of ROS involves activation of membrane-associated nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases(42). Mitochondria are a main target for arsenic-induced toxicity, and arsenic can generate ROS indirectly via the above mentioned mechanisms, or directly, by causing condensation of the mitochondrial matrix and by opening of permeability transition pores(42). One of the reasons why the brain is particularly vulnerable to oxidative stress is its high oxygen consumption: In fact, it consumes one fifth of the body's oxygen supply. Related to this, is its high energy demand and in the production of adenosine triphoshate (ATP), superoxide radicals are formed. Another reason for its vulnerability is its high content of easily peroxidiable, polyunsaturated fatty acids(155). Increased lipid peroxidation in the brain and other organs due to ROS is caused by the combined effects of GSH depletion, and reduced

activity of antioxidant enzymes like for example catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR)(156). Furthermore, the brain has an increased vulnerability to oxidative stress because neurons contain comparably low levels of radical scavengers and protective enzymes like, for example, glutathione peroxidase, GSH, vitamin E and catalase. In addition, the CNS has high concentrations of transition-metals like for example iron (Fe), that are redox-active and that, on their own, can generate ROS through catalytic processes(155). Irrespective of origin, ROS, accumulating in the intracellular space, will interact with biomolecules and modify gene expression by activating cell signalling pathways. Downstream of generation of ROS, inflammation and apoptosis can be induced(157).

3.1 BDNF AND FUNCTIONS OF NEUROTROPHINS

BDNF has recently been recognized as a biomarker to assess brain development and cognitive function(158). In addition, previously, BDNF has been suggested as an effect biomarker in epidemiological studies of neurobehavioural effects of exposure to endocrine disruptors like for example bisphenol A, poly-aromatic hydrocarbons and heavy metals(159–163). The HBM4EU project has conducted comprehensive literature searches to identify effect biomarkers of environmental neurotoxicity used in epidemiological and experimental studies(132,161,164). Among these, BDNF has been identified as one of the most promising(161). In most vertebrates, BDNF is critically important for brain development and evidence for this comes mainly from research focusing on mammals(165).

Furthermore, the importance of BDNF for neuronal network formation and synaptogenesis is illustrated by the fact that BDNF forms part of three existing AOPs (AOPs 12(136), 13(149) and 54(138)), recognized and endorsed by the Organisation for Economic Co-operation and Development (OECD)(167). However, in none of these pathways, is arsenic currently listed as a potential stressor. BDNF belongs to the class of neurotrophins, who share structural and functional similarities, and BDNF is broadly expressed in both the developing and the mature CNS. Furthermore, its release is partly controlled by neuronal activity(168). This is illustrated by the fact that, in rodents, BDNF expression reaches high levels during the weaning period, which is when neuronal circuits in the cortex mature, both structurally and functionally. As a contrast, prior to this developmental stage, BDNF expression is comparatively low(165). The highest levels of BDNF expression can be found

in the hypothalamus, hippocampus, cortex, striatum and amygdala(158,169). In addition, during early development, neurotrophins like BDNF are important for survival, growth and differentiation of CNS neurons, and are highly expressed(158).

BDNF is important for neuronal circuit development, synaptic plasticity, plasticity of neuronal networks, brain architecture, formation of brain neuronal morphology as well as maintenance this morphology (169–174). In this context, BDNF is crucially important for the regulation of brain processes related to memory and learning, in young as well as adult animals(158). Furthermore, three AOPs (AOPs 12, 13 and 54) were previously mentioned, where reduced levels of BDNF are implicated in learning and memory impairments(136,137,166). However, independently of stressors affecting these pathways, there is, as yet, no consensus about reference values for BDNF and about how low levels of BDNF must be, in order to observe adverse effects on learning and memory. Added to this comes the fact that, apart from reduced levels of BDNF, other factors and mechanisms may be involved in learning and memory impairments(166).

In the first step of its synthesis, the precursor form, pro-BDNF, is created, which is converted intracellularly to mature BDNF, or mBDNF. This maturation involves proteolytic cleavage at the synaptic cleft by the protease plasmin, which requires tissue plasminogen activator (tPA) for its activation(175). Furthermore, as a contrast to proBDNF, which is released continuously, both mBDNF synthesis and tPA release requires neuronal excitation(176). As such, dependence on neuronal excitation for its release has been shown in axon terminals as well as in dendrites(168). mBDNF has high affinity for the tyrosine kinase receptor TrkB, which mediates its biological functions, and both are broadly expressed in the brain of mammals(177).

By binding of mBDNF to TrkB, three main intracellular pathways are activated, namely, phospholipase Cγ1 (PLCγ1), Phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)(178). Furthermore, through their activation, various transcription factors are activated in the nucleus that control transcription of genes important for synaptogenesis, neuronal survival, neuronal differentiation as well as maturation and stabilization of synapses(159–161). As a contrast, proBDNF interacts with the p75 neurotrophin receptor (p75NTR) and, upon binding, the guanosine triphosphate (GTP)ase RhoA is activated. This activation, in turn, controls actin cytoskeleton polymerization,which

triggers apoptosis, inhibition of axon elongation and growth cone collapse(176,179,180). BDNF has a wide range of functions, which, partly, has to do with features of BDNF synthesis, where numerous isoforms are created, that trigger or up/downregulation of a whole range of signalling pathways by interacting with several receptors(181). Disruptions in TrkB -mediated BDNF-signalling have been shown to be associated with psychiatric disorders like for example schizophrenia(182) and major depressive disorder (MDD)(183). Furthermore, reduced levels of BDNF have been associated with autism spectrum disorders (ASDs)(184,185) as well as with negative effects on cognitive development in children(186).

3.1.1 BDNF: ANIMAL STUDIES OF ARSENIC EXPOSURE

As mentioned, were 9 animal studies of arsenic exposure identified during the literature search, using BDNF as a biomarker(**table 3**). With the exception of the study by Tyler et al. (2014)(187), was reduced levels of BDNF reported in all studies. This makes findings reported in the remaining 8 studies consistent with AOPs 13 and 54, who are both relevant for developmental neurotoxicity(137,138). In the following, the findings in all animal and *in vitro* studies will be described, and further consistencies with AOPs will be specifically noted.

Table 3. Animal studies identified during the literature search, using BDNF as a biomarker

Author	BDNF-biomarker	Tissue/matrix	Animal species	Exposure period	Exposure level and arsenic species	Main findings
Srivastava et al. (2018)	BDNF	Brain	Rat	Developmental, 28 days.	Sodium arsenite at 20 mg/kg body weight	Significantly reduced protein expression of BDNF and significantly reduced phosphorylated cAMP response element binding-protein/CREB ratio (pCREB/CREB ratio). Significantly reduced levels of pCREB phosphorylated at serine 133 (ser 133). Reduced expression of DARPP-32 and significant de-phosphorylation of Akt at serine 473 (ser 473).
Tyler et al. (2014)	BDNF	Brain	Mouse	Maternal, perinatal exposure, 7-10 days before mating	Sodium arsenate at 50µg/L drinking water	No significant changes in protein expression of BDNF and CREB. Reduced hippocampal neurogenesis and neuronal differentiation, increased baseline levels of corticosterone, blunted stress response and depressive-like behaviour
Sun et al. (2015)	BDNF	Brain	Mouse	Developmental, 3 months	"Arsenic", at 0, 1, 3 and 10 mg/ kg/ body weight	Reduced protein expression of BDNF and reduced expression of pCREB phosphorylated at serine 133 in cornu ammonis 1 (CA1) and gyrus dentatus (GD) of mouse hippocampus. Reduced object recognition

Table 3. (continued)

Author	BDNF-biomarker	Tissue/matrix	Animal species	Exposure period	Exposure level and arsenic species	Main findings
Pandey et al. (2017)	BDNF	Embryonic rat primary hippocampal neurons	Rat	Maternal and offspring, perinatal exposure from 6th. day of gestation to postnatal day 90.	Sodium arsenite, at 0.38 and 38 parts per million (ppm)	Reduced protein expression and mRNA transcription of BDNF and attenuation of the BDNF/TrkB pathway via increased bone morphogenic protein 2/ small mothers against decapentaplegic 1/5 (BMP2/Sma*d 1/5)* signalling in vitro. Exposure increased apoptosis and caused neuronal loss, decrease in neuronal perimeter as well as a decrease in dendritic length and number of dendritic branches.
Tyler et al. (2018)	BDNF	Brain	Mouse	Maternal exposure (14 days before mating) and offspring perinatal exposure until postnatal day 25.	Sodium arsenite at 50µg/L drinking water	Increases in protein expression of histone deacetylases 1, 2 and 5 (HDAC 1,2 and 5) in the frontal cortex of male, but not female mice and significant reduction in mRNA transcription of variant 4 of BDNF.
Valles et al. (2020)	BDNF and BDNF promoter methylation	Brain	Zebra-fish	Embryonal exposure, 4 hours post fertilization (hpf) to 150 days post fertilization (dpf).	Sodium arsenite at 50 and 500 µg/L	Decreased mRNA transcription of BDNF in the ancestral filial generation (F0 generation) and second filial generation (F2 generation), possibly caused by methylation of the promoter of the BDNF gene. Anxiety-like behaviours, as shown by significantly reduced exploratory behaviour, as well as altered motor activity was transmitted from the F0 to the F2 generation. Increased levels of methylation on the histone H3K4me3, which could have been involved in reducing mRNA transcription of BDNF.
Htway et al. (2019)	BDNF	Brain	Mouse	Gestational day 8-18.	Sodium arsenite at 85 mg/L drinking water	Significantly reduced levels of mRNA transcripts of the BDNF and of the serotonin receptor (5-HT5B) gene. Cognitive impairments and impaired social behaviour
Htway et al. (2021)	BDNF	Brain	Mouse	Gestational day 8-18.	Sodium arsenite at 85 mg/L drinking water	Significantly reduced levels of mRNA transcripts of the BDNF and of the serotonin receptor (5-HT5B) gene in the F2 generation. Significantly increased expression of IL-1β in the prefrontal cortex and cognitive impairments and impaired social behaviour in the F2 generation.
Chou et al. (2013)	BDNF	Neuroblastoma cells	*In vitro*	24 hours	0-20µM Sodium arsenite	Increased levels of ROS, interruption of cell cycle, reduction of mitochondrial membrane potential; reduced gene expression of BDNF as well as gene expression of n-myc downstream-regulated gene 4 (NDRG-4) and sirtuin-1 (SIRT-1). These effects of arsenic exposure were reversed by taurine administration.

3.1.1.1 Studies focusing on cAMP-responsive binding protein (CREB) and BDNF

In one of the studies identified during the literature search, Srivastava et al. (2018) observed that arsenic exposure significantly reduced expression of BDNF. In addition, it reduced the phosphorylated CREB /CREB ratio (pCREB/CREB ratio) and levels of pCREB phosphorylated at serine 133 (ser 133) in rat corpus striatum(188). Srivastava et al. (2018) suggested that this could be a result of oxidative stress caused by arsenic exposure.

Furthermore, Srivastava et al. (2018) observed that the reduction in the pCREB/CREB ratio and reduced levels of pCREB phosphorylated at ser 133 was reversed by curcumin administration(188). Due to the well-known antioxidant properties of curcumin(189), Srivastava et al. (2018) suggested that this could be caused by curcumin attenuating oxidative stress caused by arsenic exposure. Likewise, curcumin reversed the negative effect arsenic had on BDNF levels(188). This observation is consistent with findings from an earlier study, where curcumin was found to increase BDNF levels in the frontal cortex and hippocampus of diabetic mice(190). Due to the role the CREB family of transcription factors and CREB phosphorylation plays for regulation of transcription of BDNF(191), the increased pCREB/CREB ratio, which Srivastava et al. (2018) suggested was caused by curcumin administration, might have restored BDNF levels. As a contrast, Srivastava et al. (2018) did not observe any significant changes in total CREB expression as a result of arsenic exposure. In addition, Srivastava et al. (2018) reported that arsenic exposure reduced expression of the cAMP and dopamine regulated phosphoprotein DARPP-32, which is involved in modulating dopaminergic, post-synaptic signalling(188). Srivastava et al. (2018) suggested that this reduced expression could be caused by reduced levels of BDNF(188). This is due to findings in previous studies, showing reduced levels of DARPP-32 in mutant mice with a deletion of the BDNF gene(192,193). Because curcumin administration, in addition to restoring BDNF levels, also restored expression of DARPP-32, Srivastava et al. (2018) suggested that curcumin's effect on DARPP-32 was mediated by its effect on BDNF(188). In addition, Srivastava et al. (2018) observed that arsenic caused a significant dephosphorylation of Akt at ser 473(188). Because neuronal survival is thought to be mediated by BDNF, which causes phosphorylation of Akt at ser 473(58,194), this de-phosphorylation might have been caused by reduced availability of BDNF(188).

In another study identified during the literature search, Tyler et al. (2014) observed that perinatal arsenic exposure reduced hippocampal neurogenesis and neuronal differentiation in mice(187). Similar to Srivastava et al. (2018), Tyler et al. (2014) did not observe any significant changes in total gyrus dentatus expression of CREB(187). As a contrast to Srivastava et al. (2018), however, Tyler et al. (2014) did not investigate possible effects of arsenic on the CREB/pCREB ratio. Furthermore, they did not observe any significant changes in expression of BDNF. One possible explanation for this could be that Srivastava

et al. (2018) and Tyler et al. (2014) used different concentrations of arsenic in their studies: Whereas Srivastava et al. (2018) exposed rats to a concentration of sodium arsenite of 20 mg/kg body weight (bw), Tyler et al. (2014) exposed mice to drinking water laced with sodium arsenate at a concentration of 50 parts per billion (ppb) or 50µg. A concentration of 50µg arsenic in drinking water is representative for arsenic concentrations humans are exposed to through ingestion of drinking water(8). Added to this comes the fact that both metabolism of iAs as well as clearance of its metabolites has been shown to be more efficient in mice compared to humans(73). Another important difference between the studies by Srivastava et al. (2018) and Tyler et al. (2014) is that rats have a higher retention of iAs compared to mice(128), which might have affected the results. In addition to expression of BDNF and CREB, Tyler et al. (2014) also examined effects of arsenic exposure on the expression of histone deacetylase 2 (HDAC2) and the glucocorticoid receptor (GR). Here also, no effect on expression was observed. BDNF, CREB, HDAC2 and GR are all proteins implicated in hippocampal neurogenesis(187), and, despite the fact that they observed no changes in their expression due to arsenic exposure, Tyler et al. (2014) observed, as mentioned, that arsenic reduced hippocampal neurogenesis and neuronal differentiation. Tyler et al. (2014) suggested that this discrepancy could be due to arsenic affecting pathways BDNF, CREB, HDAC and GR are involved in, which, in turn, might have impaired neurogenesis(187). In addition, they observed that arsenic increased baseline levels of corticosterone, blunted the stress response and caused depressive-like behaviour(187).

Sun et al. (2015) observed, in another study identified during the literature search, reduced expression of BDNF and reduced expression of pCREB phosphorylated at serine 133 in cornu ammonis 1 (CA1) and gyrus dentatus (GD) of mouse hippocampus after exposure to arsenic at a concentration of 3 and 10 mg/kg/bw, respectively. In addition, 3 and 10 mg/kg/bw arsenic exposure reduced object recognition long-term memory (LTM)(195).

Consistencies with AOPs identified for developmental neurotoxicity

A likely consequence of the reduced hippocampal neurogenesis, which was observed by Tyler et al. (2014), is learning and memory impairments. This is because the hippocampus, in the context of memory formation, is the most widely studied brain structure(196). Thus, the reduced hippocampal neurogenesis reported by Tyler et al. (2014) might be consistent

with KE341 ("impairment, learning and memory"), involved in AOP 13 and 54 (**figure 7**). Furthermore, in both AOPs 13, and 54, reduced levels of BDNF is involved as a KE and both AOPs 13 and 54 are relevant for developmental neurotoxicity(137,138). In addition, it is likely that the reduced hippocampal neurogenesis reported by Tyler et al. (2014) is consistent with AOP42, which includes altered hippocampal anatomy (KE757), altered hippocampal physiology (KE758) and decreased cognitive function (AO402) as KEs and AO, respectively(139)(f**igure 8**). Furthermore, the association that was found by Sun et al. (2015) between arsenic exposure, reduced expression of BDNF and impaired object recognition LTM, respectively, is consistent with KE341 and AOPs 13 and 54 mentioned above (**figure 7**).

3.1.1.2 Study focusing on BDNF/TrkB and BMP2/Smad 1/5 pathways

In a study by Pandey et al. (2017), it was reported that arsenic reduced expression of BDNF and attenuated the BDNF/TrkB pathway via increased bone morphogenic protein 2/ small mothers against decapentaplegic 1/5 (BMP2/Smad 1/5) signalling *in vitro,* in embryonic rat primary hippocampal neurons (**figure 3**)(197). BMPs have several functions and belong to the transforming growth factor (TGF-β) superfamily. BMP2, along with BMP4 and 6, are expressed by hippocampal neurons, and by binding to type I (BMPR1A and BMPR1B) and type II (BMPR2) receptor subunits, signal transduction is mediated by phosphorylated Smad proteins(198–202). The findings by Pandey et al. (2017) are relevant, due to the fact that hippocampal development and function appears to be strongly affected by BMP signalling pathways(203,204). In their study, Pandey et al. (2017) observed that expression of both BMP2, the BMP2 receptor BMPR2 and Smad 1/5, that all are involved in these pathways(205), was increased due to arsenic exposure. Furthermore, exposure caused neuronal loss, a decrease in neuronal perimeter as well as a decrease in dendritic length and number of dendritic branches(197). In one part of the study by Pandey et al. (2017), cells were exposed to arsenic, which reduced levels of BDNF and increased apoptosis. Subsequently, cells were co-treated with arsenic + recombinant BDNF, which was shown to reduce the apoptosis-inducing effects of arsenic alone. In another part of the experiments, cells were co- treated with either recombinant BDNF together with arsenic or the BMP2 antagonist noggin together with arsenic, respectively. This showed that co-treatment with

recombinant BDNF + arsenic did not reduce BMP2 levels and had no effect on the BMP2/Smad pathway. Conversely, co-treatment with noggin + arsenic inhibited the BMP2/Smad pathway and was shown to restore BDNF expression. Furthermore, co-treatment with noggin restored BDNF/TrKB signalling and improved neuronal survival (**figure 3**). In addition, Pandey et al. (2017) observed that the anti-apoptotic effect of noggin was abolished by co-treatment with noggin, arsenic and the TrKB inhibitor K252a. This indicated a link between the BMP2/Smad and the BDNF/TrKB pathways: Although noggin inhibited the BMP2/Smad pathway, and therefore restored the reduced BDNF expression, K252a inhibited signalling in the BDNF/TrKB pathway, which, in turn, increased apoptosis(197). Furthermore, based on their observations, Pandey et al. (2017) suggested that signalling via the BMP2/Smad 1/5 pathway could have activated expression of gene products that may have repressed expression of BDNF(197).

To the best of my knowledge, have no other studies yet investigated the involvement of BMPs in neuronal apoptosis and reduced expression of BDNF as a result of exposure to heavy metals. The roles of BMPs in neuronal function have in earlier studies been shown to be diverse(197). For example, Luan et al. (2015) and Pei et al. (2013) have reported neuroprotective effects of BMPs(206,207). Whereas Pandey et al. (2017) observed that increased BMP2 expression was associated with reduced BDNF, it has also been shown that BMPs are able to promote BDNF expression(208,209). Conversely, BDNF has been reported to trigger BMP/Smad activation(210). A further discrepancy between earlier studies and findings by Pandey et al. (2017) is that, earlier, BDNF and BMP have been shown to complement each other in enhancing neuronal function and reducing neuronal stress(210,211). Instead, Pandey et al. (2017) observed neuronal dysfunction and apoptosis and suggested that the observed increase in BMP2/Smad 1/5 signalling and the reduced BDNF signalling could be specific for hippocampal neuronal apoptosis associated with exposure to arsenic or other heavy metals(197).

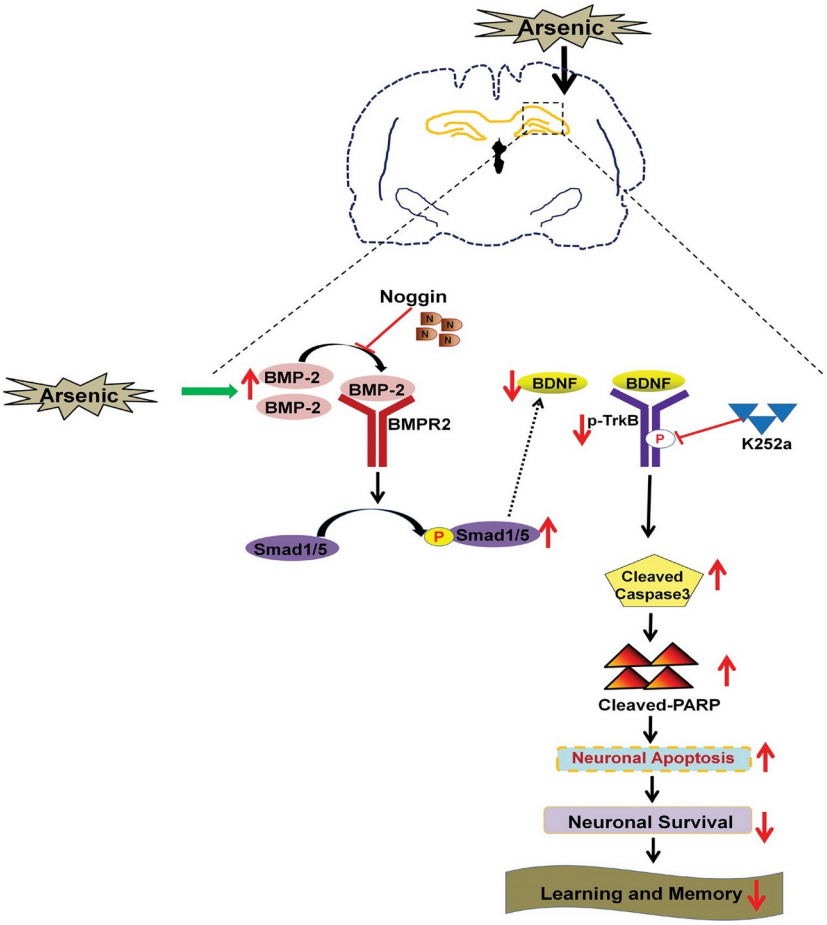

Figure 3. (taken from Pandey et al. (2017) with permission). Increased hippocampal apoptosis due to arsenic exposure mediated by an increase in expression of BMP2, which impaired BNDF/TrkB signalling. Arsenic caused activation of the BMP2 pathway by increasing expression of BMPR2 and BMP2, as well as by enhanced p-Smad1/5 signalling. Increased BMP2/Smad signalling reduced expression of BDNF and impaired signalling in the BDNF/TrkB pathway, leading to apoptosis of hippocampal neurons. The BMP2/Smad signalling was blocked by noggin, which restored expression of BDNF and signalling in the BDNF/TrkB pathway (197).

Consistencies with AOPs identified for developmental neurotoxicity

Pandey et al. (2017) reported, as mentioned, that arsenic exposure caused neuronal loss, a decrease in neuronal perimeter as well as a decrease in dendritic length and number of dendritic branches(197). These findings, as well as the reduced expression of BDNF reported by Pandey et al. (2017), are consistent with AOP13: In AOP13, which is relevant for developmental neurotoxicity, the KE "reduced levels of BDNF" (KE381) is adjacent to the KE "abberrant dendritic morphology" (KE382), which, in turn, leads to decrease in neuronal network function (KE386). In addition, AOP13 describes how reduced levels of BDNF leads to cell injury or death (KE55)(137), which also was reported by Pandey, et al. (2017). Furthermore, observations by Pandey et al. (2018) are consistent with observations

from other studies: In immortalized cell lines, for example, arsenic has been reported to reduce neurite outgrowth(55,57), and impaired neurite outgrowth due to arsenic exposure has been associated with cell death of cortical neurons(56,212). Together, these findings strengthens the probability that AOP13 is relevant for arsenic-mediated neurotoxicity. In addition, the neuronal loss, decrease in neuronal perimeter as well as decrease in dendritic length and number of dendritic branches reported by Pandey et al. (2017) may be consistent with AOP54 (**Figure 8**). In AOP54, reduced levels of BDNF is involved as a KE (KE381) and AOP54 describes how reduced levels of BDNF leads to a decrease in neuronal network function (KE386) and reduced synaptogenesis (KE385) which, in turn, leads to learning and memory impairment as an AO (AO341)(138).

3.1.1.3 Studies focusing on epigenetic regulation, transgenerational effects and serotonergic neurotranmission

Histone modifications and BDNF, methylation of the promoter region of the BDNF gene

Tyler et al. (2018) observed a small reduction in expression for variant 3 and a significant reduction for variant 4 BDNF expression in the frontal cortex of male, but not female mice as a result of developmental arsenic exposure. In addition, increased expression HDAC5 was reported. Tyler et al. (2018) suggested that this increased expression caused an increase in histone deacetylation which, in turn, repressed BDNF expression in males(213). The sex-dependent adverse effects of arsenic exposure reported by Tyler et al. (2018) are consistent with findings from earlier studies, indicating that the female cortex is less vulnerable to damage caused by some toxicants than the male cortex(214,215). In addition, it has been shown that males and females respond differently to developmental arsenic toxicity(24,216). However, the mechanisms that might protect females more than males against arsenic exposure, are still unknown(213)**.**

In an animal study, Valles et al. (2020) reported trans-generational changes as a result of arsenic exposure that manifested themselves in a decreased expression of BDNF in the ancestral filial generation (F0 generation) and second filial generation (F2 generation) of zebrafish. Valles et al. (2020) suggested that this was caused by methylation of the promoter of the BDNF gene, and observed that this methylation increased dose-dependently(217). A similar increase in the methylation of the promoter region of the BNDF gene has previously

been reported by Li et al. (2020) in zebrafish exposed to an organophophorous flame-retardant. Similarly, in the study by Li et al. (2020), reduced expression of BDNF was also observed(218). However, it is important to notice that the regulation of BDNF expression is complex: Its expression has been shown to be affected by methylation of different promoter regions (219). In addition, expression may be regulated by histones like for example H3K4me3 and H3K27ac(220) as well as by non-coding RNAs(221). Thus, it might be problematic to use methylation of the BDNF promoter region as a reliable indicator of changes in the levels of BDNF. In their study, Valles et al. (2020) also observed that anxiety-like behaviours, as shown by significantly reduced exploratory behaviour, as well as altered motor activity, was transmitted from the F0 to the F2 generation(217). Valles et al. (2020) suggested that this transmission of behaviour could, in part, have been caused by epigenetic disruption of BDNF-TrkB signalling(217,222). In addition, they suggested that epigenetic dysregulation of glucocorticoid signalling mediated by disruption of the serotonergic system could be responsible for this behavioural transmission(217,223). Valles et al. (2020) also reported increased levels of methylation on H3K4me3, which they suggested could have been involved in reducing expression of BDNF in the male and female F0 and F2 generation(217). H3K4me3 is an epigenetic biomarker which has been used as an indicator of transcriptional activation of genes associated with neuronal activity as well as synaptic transmission(224). Furthermore, it is generally agreed that there is a positive correlation between levels of H3K4me3 and gene expression(225). However, H3K4me3 also *reduces* gene expression by recruiting suppressors of transcription(226). Therefore, Valles et al. (2020) suggested that the increased levels of H3K4me3 methylation could have been implicated in causing the reduced expression of BDNF that was observed in the male and female F2 generation of zebrafish(217).

The serotonergic system and BDNF

Htway et al. (2019) observed significantly reduced levels of mRNA transcripts of the BDNF and of the serotonin receptor (5-HT5B) genes in mice after exposure to 85 mg/L of sodium arsenite during gestation(227). Htway et al. (2019) suggested that the cognitive impairments and impaired social behaviour that also was observed, was associated with injury to cells within the prefrontal cortex(227). This is due to the role the prefrontal cortex plays for social behaviour(228). In addition, the reduced expression of the serotonin receptor

that was reported might have indicated that serotonergic neurotransmission was affected by arsenic exposure(227). Furthermore, impaired serotonergic neurotransmission and reduced levels of BDNF might, in part, have contributed to the impaired social behaviour Htway et al. (2019) observed(227). This is due to the role serotonergic neurotransmission plays in mood disorders which, in turn, affects social behaviour(229,230) and the role BDNF plays for learning and memory, which also is important for social behaviour(231). In this context, it might be relevant that Tyler et al. (2018) and Sun et al. (2015) reported learning impairments in the previously mentioned studies, as well as reduced expression of BDNF(195,213). Htway et al. (2019) suggested, as mentioned, that neurons in the prefrontal cortex were damaged due to arsenic exposure, and that these damages could have been associated with reduced expression of the 5-HT 5B receptor and thus, impaired serotonergic neurotransmission(227). This is due to the neuromodulatory effect of serotonin(227). 5-HT 5B is an inhibitory serotonin receptor(232) and, because of its reduced expression, Htway et al. (2019) suggested that pyramidal cells were hyper-exited as a result of glutamatergic signalling, which ultimately lead to excitotoxic cell injury within the prefrontal cortex. The decreased levels of BDNF that also were observed, might have exacerbated the cell injury, due to BDNFs neuroprotective effects(227). Furthermore, in the study by Htway et al. (2019), it was assumed that the impaired serotonergic neurotransmission could be linked to the reduced expression of BDNF. This is because expression of BDNF is strongly controlled by serotonergic neurotransmission and, conversely, BDNF is important for both function as well as development of serotonergic neurons(233).

The serotonergic system and BDNF: Transgenerational effects

Htway et al. (2021) reported results that were similar to those reported by Htway et al. (2019). One of the differences was that, whereas Htway et al. (2019) examined adverse effects of maternal arsenic exposure on offspring of mice, Htway et al. (2021) investigated effects on the F2 generation of mice born to gestationally exposed mice (F1 generation) (232). Similar to Htway et al. (2019), Htway et al. (2021) reported that F2 mice had a significantly decreased mRNA expression of the 5-HT 5B receptor at the age of 41 and 74 weeks and a significantly decreased mRNA expression of BDNF at the age of 74 weeks in the prefrontal cortex(232). In addition, similar to Htway et al. (2019), who observed impaired social behaviour in the F1 generation, this was also observed in the F2 generation

by Htway et al. (2021)(232). However, despite of the transgenerational effects that were observed, Htway et al. (2021) was not able to prove that they were due to epigenetic inheritance. In addition, expression of the pro-inflammatory cytokine IL-1β in the prefrontal cortex was significantly increased in F2 mice at the age of 41 weeks and the oxidative stress marker HO-1 was significantly increased in all F2 mice. However, Htway et al. (2021) was not able to prove that the increases in oxidative stress and neuroinflammation were correlated with increased apoptotic cell death(232). Htway et al. (2021) suggested that the increased expression of HO-1 was a result of apoptotic cell death due to excessive glutamatergic signalling(232). On it own, this cell death might have contributed to reduce levels of BDNF.

3.1.1.4 n-myc downstream-regulated gene 4 (NDRG-4) and BDNF

In an intervention study, Chou et al. (2013) reported that expression of n-myc downstream-regulated gene 4 (NDRG-4) and BDNF were reduced in human neuroblastoma SH-SY5Y cells treated with arsenic(141). Because NDRG-4 deficient mice have been reported to have significantly reduced cortical gene expression of BDNF(234), Chou et al. (2013) suggested that expression of BDNF was dependent on expression of NDRG-4. Furthermore, administration with taurine restored expression of both BDNF and NDRG-4(141). Due to the well-known antioxidant properties of taurine(235–237), Chou et al. (2013) suggested that the effects on BDNF and NDRG-4 were mediated by a reduction in oxidative stress(141). Furthermore, arsenic reduced expression of sirtuin-1 (SIRT-1)(141). Because activation of SIRT-1 has been shown to trigger expression of proteins involved in protection against oxidative stress and apoptosis(238), Chou et al. (2013) suggested that its downregulation was implicated in increasing cellular oxidative stress. Conversely, treatment with taurine restored expression of SIRT-1(141).

3.1.1.5 Limitations of animal studies

All animal studies, focusing on effects of arsenic exposure on BDNF identified during the literature search, used brain tissue to measure levels of BDNF. Therefore, they do not offer any evidence for the detectability of effects of arsenic exposure on BDNF using minimally invasive measurement methods. Another possible limitation with the studies identified, is that the majority used exposure concentrations that are too high to be representative for

concentrations humans are exposed to environmentally. As a consequence, effect biomarker responses reported in experimental studies may not be translatable to human studies.

3.1.2 BDNF: HUMAN STUDY OF ARSENIC EXPOSURE

In the only human study identified during the literature search (**table 4**), focusing on effects of developmental arsenic exposure on BDNF, Rodríguez-Carrillo et al.(2022) reported a significant association between arsenic exposure and increased methylation of the BDNF gene at CpG5 as well as total CpGs methylation percentage, respectively, among adolescents(239). 5'-C-phosphate-G-3' (CpG) sites are parts of the DNA where cytosine and guanine are separated by one phosphate group in the 5' to 3' direction of the base sequence. These CpG sites are present with high frequency in regions of the genome termed "CpG islands" and, in humans, CpG islands are found in around 70% of promoters situated close to gene transcription start sites(240,241). Rodríguez-Carrillo et al.(2022) justified using blood methylation levels of the BDNF gene at a total of 6 CpGs, based on a study by Kundakovic et al. (2015). In this study, it was shown that levels of methylation of the BDNF gene at 6 CpGs, measured in blood, mirrored BDNF transcription levels and methylation pattern in mouse hippocampus. Based on this, Kundakovic et al. (2015) proposed using blood measurements of levels of BDNF DNA methylation as a substitute biomarker for brain expression of BDNF in humans(160). In addition, Rodríguez-Carrillo et al.(2022) found an association between urinary arsenic levels and reduced levels of serum BDNF(239). This was, in part, suggested to be due to increased methylation of the BDNF gene, and in their study, it was found a non-linear association between urinary arsenic levels and somatic complaints, anxiety and thought disorders (internalizing behaviour)(239). The findings by Rodríguez-Carrillo et al.(2022) are consistent with results from an earlier human study by Karim et al. (2019), showing that arsenic exposure was associated with significantly reduced serum BDNF levels in adults. In addition, in the study by Karim et al. (2019), arsenic exposure was significantly associated with cognitive impairment(69).

Table 4. Summarized data from the human study by Rodríguez-Carrillo et al.(2022)

Author	Study type	Number, age and gender of participants	BDNF biomarkers	Blood and/ or urinary As levels	Main findings	Adjustment factors
Rodríguez-Carrillo et al. (2022)	Cross-sectional	125 male adolescents, mean age: 16.9 years	Serum BDNF protein and methylation of the BDNF gene	Urine median value: 24.20 µg/L	Significant association between arsenic exposure and increased methylation of the BDNF gene at CpG5. Association between urinary arsenic and reduced serum BDNF. Non-linear association between urinary arsenic levels and somatic complaints, anxiety and thought disorders (internalizing behaviour).	Exposure to passive smoking, maternal education level and maternal intelligence. Fish consumption, body mass index (BMI), age of participating adolescents and exposure to other metals.

3.1.2.1 Strengths and limitations of the human study by Rodríguez-Carrillo et al. (2022)

One limitation with this study was the small number of participants (n= 125), weakening its statistical power. Another, was that it was a cross-sectional study, precluding the ability of linking causes to effects. Yet another limitation is that Rodríguez-Carrillo et al. (2022) conducted no speciation analysis. Despite the fact that fish consumption was a variable that was adjusted for, various other potential sources of arsenic exposure were not investigated. Such an investigation might have made it possible to assess the impact of exposure to inorganic arsenic and organoarsenicals, respectively, on neurobehaviour, BDNF DNA methylation and serum BDNF protein levels. In their study, Rodríguez-Carrillo et al. (2022) measured concentrations of inorganic and organic As in urine. The advantages of urinary measurements are obvious, due to their non-invasive nature and ease of sampling, which are both important factors in large epidemiological studies and in cases where repeated measurements are used. Furthermore, another benefit is that, using urinary measurements, it may be possible to measure levels of a wider range of arsenic metabolites, arising from exposure to drinking water and foodstuffs, respectively(242). In addition, urine is believed to be a suitable effect biomarker of arsenic exposure, because urinary arsenic concentrations are maintained at relatively stable levels, provided dietary patterns are consistent(243,244). Urinary measurements might, however, not have the same accuracy achieved by combined blood and urinary measurements.

One of the factors corrected for in the study by Rodríguez-Carrillo et al. (2022) was exposure to passive smoking. This is due to the adverse effects passive smoking and maternal smoking during pregnancy can have on neurodevelopment(245–247). Furthermore, it was corrected for maternal education level and results of tests of maternal intelligence. This is because such variables can affect neurodevelopment. As such, these factors have been extensively accounted for in epidemiological studies assessing neurodevelopmental

outcomes(248,249). Furthermore, fish consumption was, as mentioned, corrected for, because fish, despite its content of for example omega-3, which is believed to be beneficial for neurodevelopment, also is a source of exposure to As as well as mercury and lead(250,251). In addition, it was corrected for body mass index (BMI) and age of participating adolescents. This is because BMI is correlated with behaviour in children. Furthermore, age differences, even in months, is correlated with differences in stages of brain development in children and adolescents (252,253).

Rodríguez-Carrillo et al. (2022) examined neurobehavioural effects and effect on BDNF of exposure to mercury, cadmium and lead, in addition to arsenic. To assess effects of exposure to single metals, regression models were used. On the one hand, such models can only provide an estimation of the isolated effect of arsenic on BDNF and neurobehavioural parameters. On the other, environmental exposure to multiple metals represents a realistic scenario for humans, as opposed to single-metal exposure(254). In addition to metals, humans are exposed to multiple other environmental toxicants that may affect BDNF DNA methylation and serum BDNF protein levels(11), and in their study, Rodríguez-Carrillo et al. (2022) did not adjust for this background exposure. One example of such a chemical is bisphenol A (BPA), which is ubiquitous in the human environment. Furthermore, in this context, it is relevant that BDNF is regarded as a promising effect biomarker for human exposure to BPA(161).

3.1.3 ARSENIC EXPOSURE AND BDNF: OTHER POSSIBLE MECHANISMS

3.1.3.1 Possible effects of arsenic exposure on BDNF, mediated by NMDARs

Rodríguez-Carrillo et al. (2022) suggested that arsenic's effect on BDNF could, in part, be mediated by its effects on NMDAR. In the developing nervous system, activation of the NMDAR causes increased release of BDNF(255,256). NMDAR-activation and BDNF-release is associated with long-term potentiation (LTP) and, with that, memory formation, synaptic plasticity as well as strengthening of synapses in the hippocampus(256). Evidence for this NMDAR-dependent LTP induction has been found in the developing brain of rodents, and the significance of NMDAR for LTP in the developing nervous system is partly due to the NMDAR subunits that dominate in the developing CNS as opposed to the adult CNS(256–258). The importance of the NMDAR for learning and memory formation is both

thoroughly documented and well accepted (259). This is illustrated by experiments with NMDAR antagonists, and mutant mice lacking NMDAR subunits have been shown to have impaired LTP(260).

During the literature search, no animal studies were identified, where developmental arsenic exposure was reported to affect NMDARs and BDNF, respectively. However, due to the importance of NMDARs for BDNF release(255,256), it is relevant to mention one animal study by Ramos-Chávez et al. (2015). Here, gestational arsenic exposure was reported to cause downregulation of the hippocampal NMDAR subunit NR2B in mice and this was associated with impaired spatial memory(113). Ramos-Chávez et al. (2015) suggested that increased oxidative stress as well as excessive intracellular glutamate could, in part, be responsible for altering expression of NMDAR subunits(113,261).

3.1.3.2 Possible effects on BDNF mediated by inflammation

Rodríguez-Carrillo et al. (2022) suggested, in the already mentioned study, that arsenic's effects on BDNF, in part, could be mediated by its effect on neuroinflammation and oxidative stress(149). Arsenic exposure might, as mentioned, trigger generation of ROS and cause depletion of GSH, which can cause cell damage or death(39). Furthermore, it is known that cell damage or death, on its own, may cause neuroinflammation, which can reduce levels of BDNF by damaging, for example, cells in the hippocampus(262). In addition, a large body of evidence exists for links between systemic inflammation and inflammation in the brain, or neuroinflammation(263). In the context of arsenic exposure, both *in vitro* and *in vivo* studies have shown that low-level arsenic exposure is capable of inducing strong, pro-inflammatory responses(264,265), which may arise dependently or independently of oxidative stress(39). Such effects can be observed, even at levels of exposure via drinking water, that are characterized as low by current regulatory standards(265). Furthermore, cytokines and other inflammatory mediators in systemic circulation produced by such responses, may cross the BBB by active transport, and, at places where the BBB is not intact, diffusion is a possible transport mechanism. Other mechanisms, by which cytokines and other inflammatory mediators might gain access to the brain, are via activation of vagal afferents, and cytokine signal propagation(266).

Independently of heavy metal exposure, studies have shown that inflammation can reduce levels of BDNF(262). Intraperitoneal injection of lipopolysaccharide (LPS) or interleukin-1β (IL-1β) in rats, for example, caused a significantly reduced expression of hippocampal BDNF(267). Furthermore, inflammation may affect expression of the various BDNF transcripts: For example, in rats exposed to *E.Coli*, CA1 of the dentate gyrus expression of exon I, II and IV of BDNF was reduced(268). Possibly, this shows that inflammation disrupts expression of distinct isoforms of BDNF(262).
Inflammation is thought to play an important role for mood disorders(263). Convincing in this context, are the results from a Danish study with 73.131 participants, that showed that members of the general Danish population with low-grade depressive symptoms, had significantly elevated C-reactive protein (CRP) concentrations in blood compared to those without these symptoms(269). Possibly, these effects were mediated by BDNF. Furthermore, in several human and animal studies that have shown correlations between depression or depressive-like behaviours and immunological or inflammatory alterations, simultaneous changes in BDNF expression and function have been reported(262). These changes in BDNF expression and function are not surprising, due to the fact that BDNF is well known to be implicated in for example major depression and other psychiatric disorders. Likewise, it is well known that a positive response to treatment causes levels of BDNF to increase(270,271). However, despite the strong links between reduced levels of BDNF and inflammatory conditions, and their well known associations with depression and other mental disorders, the mechanisms by which pro-inflammatory cytokines mediate these effects, are still unclear(262).

3.1.3.3 Inflammation and the tryptophan (TRP)/ kynurenine (KYN) pathway: Possible effects on serotonin and BDNF

Rodríguez-Carrillo et al. (2022) also suggested that arsenic's effects on BDNF, in part, could be mediated by its effect on serotonin(149). The interaction between serotonergic neurotransmission and BDNF is, as already mentioned, reciprocal: Whereas expression of BDNF is strongly controlled by serotonergic neurotransmission, BDNF is important for both function as well as development of serotonergic neurons(233). Pro-inflammatory cytokines (for example tumor necrosis factor-α (TNF-α) and interferon-γ [IFN-γ]) can strongly trigger

the activation of the enzyme indoleamine 2,3- dioxygenase (IDO)(272). IDO is part of the tryptophan (TRP)/ kynurenine (KYN) pathway, and abnormalities in this pathway have been linked to several neuropsychiatric disorders(272). The tryptophan (TRP)/ kynurenine (KYN) pathway catalyses TRP hydrolysis, which leads to the production of numerous intermediates(273). TRP is an essential amino acid, which is a raw material for protein synthesis. In addition, it is a necessary precursor for the production of serotonin. Around 95% of TRP is degraded in the liver via the KYN pathway, whereas the remaining 5% is used for synthesis of serotonin(273). TRP is oxidated via IDO and tryptophan dioxygenase (TDO) and, normally, only a marginal fraction of TRP is degraded by IDO(274). During inflammatory conditions, however, pro-inflammatory cytokines can, as mentioned, strongly induce IDO **(figure 4)** (272) . Furthermore, since IDO is expressed in the brain, it is possible that TRP-depletion will negatively affect serotonin synthesis and serotonergic neurotransmission taking place there(274). In addition, through its effects on the inflammatory response, it is likely that arsenic causes serotonin levels to decrease, via an increased activation of IDO and TRP hydrolysis(39). One human study of arsenic exposure might offer indications of this: Mukherjee et al. (2014) reported increased levels of platelet P-selectin and suggested that this upregulation may have played a role in causing inflammation among women exposed to arsenic(275). This is because P-selectin facilitates neutrophil transmigration(276). This inflammation, in turn, was suggested to cause upregulation of IDO and reduced levels of serotonin(275).

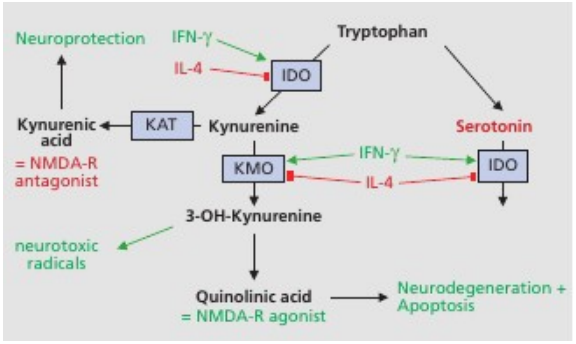

Figure 4**.** *Metabolic pathways of tryptophan and kynurenine and how its metabolites might be implicated in neurodegeneration. Pro-inflammatory cytokines can strongly induce indoleamine 2,3- dioxygenase (IDO), leading to serotonin depletion via increased TRP hydrolysis. KAT: kynurenine aminotransferase I, NMDA-R: N-methyl-d-aspartate receptor, KMO: kynurenine 3-monooxygenase. Taken from Müller (2017) with permission (272)).*

3.1.3.4 Thyroid hormones and BDNF

AOP54 describes how decreased TH synthesis (KE277) leads to reduced levels of BDNF (KE381), which, in turn, leads to learning and memory impairment as an AO. Furthermore, as will be described later, are THs essential for brain development(138) and arsenic is known to disrupt TH homeostasis in humans and animals(24,70). The developmental window, within which hypothyroidism causes damage to the CNS as well as the nature of these damages, suggest that the effects of THs on neurodevelopment are mediated by neurotrophins(277). Among these, it is likely that BDNF plays a main role, due to its crucial role in development of the CNS(278). Furthermore, numerous studies have shown regulatory effects of THs on expression of BDNF in the brain and that these effects, in turn, affected neurodevelopment(279–281).

The hippocampus and the neocortex are among the brain regions known to have the highest expression of BDNF(282). In addition, these two brain regions are essential for learning and memory(277). In this context, it is relevant that thyroid insufficiency has, not only been shown to reduce mRNA and protein levels of BDNF in the developing brain, but also that the hippocampus and cortex were the brain regions most probably affected by such reductions(287,288).

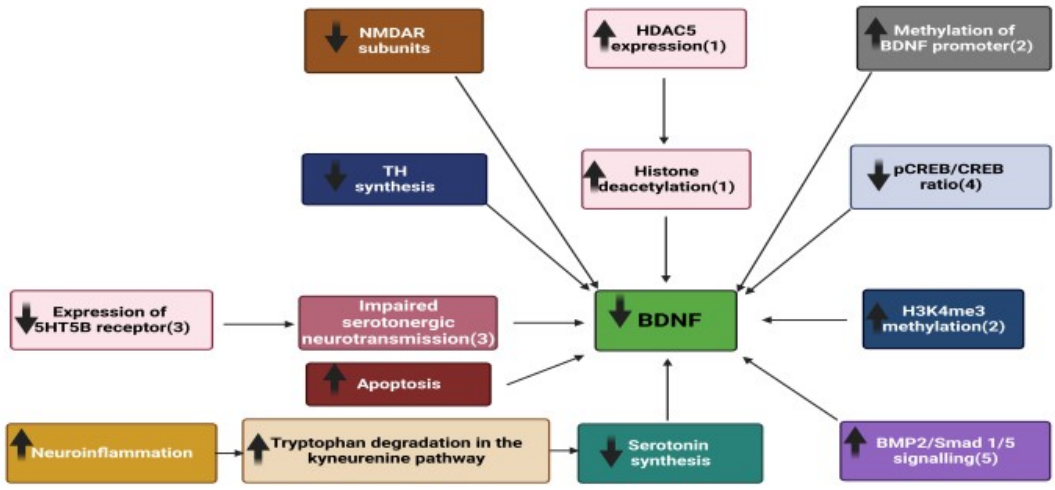

Figure 5. Graphical summary of mechanisms suggested in experimental studies identified during the literature search, by which arsenic may reduce levels of BDNF: (1), increased histone deacetylation and HDAC5 expression (Tyler et al. (2018)), (2), increased H3K4me3 methylation and methylation of BDNF promoter (Valles et al. (2020)), (3), decreased expression of 5HT5B receptor, leading to impaired serotonergic neurotransmission (Htway et al. (2019)), (4), reduced pCREB/CREB ratio (Srivastava et al. (2018), (5), increased BMP-2/Smad 1/5 signalling (Pandey et al. (2017). In addition, in **Figure 5.,** mechanisms suggested in other experimental studies, not identified during the literature search, are shown: Increased neuroinflammation, leading to increased tryptophan degradation in the kyneurenine pathway and decreased serotonin synthesis; increased apoptosis, decreased expression of NMDAR subunits and decreased TH synthesis.

3.1.4 MEASUREMENT OF BDNF

BDNF has previously been measured in cerebrospinal fluid (CSF)(285), platelets, plasma, serum and whole blood(286). Due to the fact that mBDNF is taken up by platelets in humans, it is distributed to all tissues and organs(287). Furthermore, similar to endothelial cells, dendritic cells, and eosinophils, it has been reported that lymphocytic cells have expressed BDNF *in vitro.* As yet, no OECD guidelines have been developed for the measurement of BDNF(165). The measurement of changes in brain levels of BDNF is technically challenging. This in, in particular, true when these changes are small. In such cases, it is difficult to identify differences that are significant(165). In humans, BDNF can be measured in cerebrospinal fluid (CSF) with commercially available enzyme-linked immunosorbic assays (ELISAs)(286). In addition, there exists immunobead-based multiplex assays, enabling high throughput screening of BDNF CSF levels(285). Apart from this, BDNF can be measured in whole blood, serum, plasma and platelets. For such measurements, there are several double antibody sandwich ELISA kits commercially available (286). Furthermore, a study by Klein et al. (2011) has shown that blood levels of BDNF reported in humans, in most cases, are comparable to blood levels of BDNF reported in animals, and that hippocampal and blood BDNF levels are positively correlated in rats and pigs(268).

3.2 THYROID HORMONES (THs)

3.2.1 Synthesis and homeostasis of THs

It is generally agreed, that altered serum levels of TH can be used in the diagnosis of thyroid disease or to diagnose disruption of TH homeostasis as a result of exposure to chemicals or toxicants(288). Thyrotropin-releasing hormone (TRH), which is released from the hypothalamus, stimulates the pituitary gland to synthesize and release thyroid stimulating hormone (TSH). TSH acts on the thyroid gland by stimulating it to take up iodine from the bloodstream, which is used in the synthesis of thyroxine (T4) and triiodothyronine (T3). The thyroid gland is the source of ca 20% of T3, and is the only place in the body where synthesis of T4 takes place(289). The various iodothyronines are created by inner or outer ring monodeiodination of T4 in sequential order, by the deiodinase enzymes DIO1, DIO2 and DIO3 in various organs(290). Furthermore, because these enzymes are dependent on selenium, selenium deficiency can contribute to hypothyroidism in humans(291). In

humans, DIO1 and DIO2 convert T4 into T3, which binds more easily to the TR than T4, due to higher affinity(292). Only a small fraction (less than 1%) of the released T3 and T4 is freely available and not bound to transport proteins. This free T3 and T4 (fT3 and fT4) is biologically active, and, in the body, there is an equilibrium between free and bound T3 and T4, respectively(138).

The Na+/I- symporter (NIS) is a membrane-bound glycoprotein responsible for the first step of TH synthesis, namely, uptake of iodine from the bloodstream into the thyroid follicular cells. In addition, NIS is a well-known target of several chemicals that, by inhibiting NIS, causes TH synthesis to decrease(138). This inhibition will, in turn, reduce the release of TH into the bloodstream and thus, reduce its availability in the brain. Subsequently, this might affect brain development, cognition and behaviour in children(138).

Thyroid peroxidase (TPO) is the most important enzyme for thyroid hormone synthesis. TPO is located in thyroid follicular cells and acts by oxidating and converting iodide (I^-) to an intermediate product, which is thought to be either hypoiodate or iodinium. This intermediate iodide product is subsequently integrated in thyroglobulin-associated tyrosyl residues in the thyroid follicles. In the next step, TH is produced by conjugation of two iodotyrosyl residues, in a reaction catalyzed by TPO(294,295). If TPO is inhibited, for example through exposure to environmental toxicants, levels of thyroid hormones will be reduced. Via feedback mechanisms, this will trigger an increase in the secretion of TRH and TSH(293). TH production in the thyroid gland and levels of THs in the blood are controlled by the Hypothalamus-Pituitary-Thyroid (HPT) axis (**figure 6**).

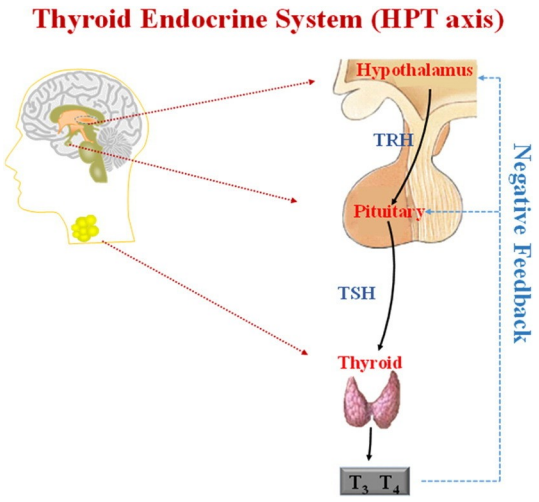

Figure 6. *Regulation of TH homeostasis through the HPT axis. Taken from Sun et al. (2016) with permission(296).*

Involved in this regulation are: 1) release of TRH in the hypothalamus, 2) secretion of TSH from the anterior pituitary, 3) transport of hormones via transport binding proteins, 4) uptake of THs by the cells, 5) intracellular regulation of TH concentration by deiodinases and 6) the nuclear thyroid hormone receptor (TR). In the fetus, regulation of TH homeostasis is, in addition, regulated by 7) passage of T4 and T3 across the placenta(297). T4 is transferred from serum to the brain via the vascular endothelia and, subsequently, into the astrocytes. In astrocytes, conversion of T4 to T3 by deiodinase takes place, and T3 is in the next step taken up by neurons(297). T3 interacts with the TR and, subsequently, TR and the retinoid X receptor (RXR), in most cases, form a heterodimer, which functions as a transcription factor. By binding of this heterodimer to thyroid response elements (TREs), transcription of target genes is either activated or repressed(298).

3.2.2 Thyroid hormones, brain development and brain function

THs were during the literature search identified as potential effect biomarkers of arsenic-mediated neurotoxicity. Because this master's thesis focuses on developmental neurotoxicity, it would might have seemed logical that it was focused exclusively on studies investigating effects of arsenic exposure on children, adolescents and young animals, respectively. However, due to the well-recognized fact that maternal (and thus, adult) TH disruption can have adverse effects on neurodevelopment in children and young animals(299–306), and because arsenic exposure is, as will be described later, associated with maternal hypothyroidism in both human and animals(24,70), studies focusing on arsenic's effects on TH homeostasis in adult humans and adult animals were included among studies selected and identified during the literature search.

In AOP 54 and KER1506, decreased TH synthesis (KE277), leading to decreased availability of TH in the blood and, consequently, also in the brain, is a KE related to learning and memory impairments (AO341)(138,307). Although AOP54 and KER 1506 do not list arsenic as a stressor, it is, as will be described further, likely that arsenic’s effects on for example learning and memory are mediated by its effects on TH synthesis. For both KER1506 and AOP54, as well as the stressors recognized for AOP54 and KER1506, there

exists a strong weight of evidence(138,307). The brain development is particularly vulnerable to TH disruption, and this fact has been recognised for several decades(301,302). Numerous human studies have shown that reduced levels of maternal TH in peripheral circulation can cause learning and memory impairments in children, as well as other neurophysiological deficits(299,300). In animal studies, it has for example been shown reduced dendritic arborization and a reduction in the number of synapses of pyramidal neurons in hypothyroid animals(308,309), which likely was associated with cognitive impairment. In addition, animal studies have shown that synaptic plasticity is impaired in offspring due to maternal hypothyroidism(303–306).
After around 12 weeks of gestation in humans, the fetal thyroid gland starts it own production of TH, but prior to this, the fetus relies on maternal TH. The first trimester (first 12 weeks of pregnancy) is therefore a period when the fetus is most vulnerable to maternal TH deficiency(310). TH is essential during the whole period when the brain develops in humans, but its role is most critical during the perinatal period of development(311). In the third trimester, there is for example a rapid increase in synaptogenesis and in this period, around 40.000 synapses are being formed per minute(312). Two brain regions, both critically linked to cognitive function, and whose development are dependent on THs, are, among others, the neocortex and the hippocampus(11). Animal studies have for example shown associations between reduced levels of serum THs and altered excitatory and inhibitory transmission in the hippocampal gyrus dentatus and cornu ammonis 1 (CA1) during the perinatal period. Such effects might be irreversible, since they have been reported to remain into adulthood and after TH levels were normalized(303–306,313–315).

In the next section, one animal study identified during the literature search, will be presented. In this, it was shown that arsenic exposure significantly reduced expression of the calcium/calmodulin-dependent protein kinase type IV (CaMKIV) protein and significantly reduced expression of the thyroid receptor gene β (TR β) and the TR β1 protein in the cerebellum of mice(142). CaMKIV and the pathways it is part of, are essential for brain development, brain function and, with that, learning and memory(316). In addition, expression of CaMKIV has been shown to be specifically induced by T3(316). By formation of a complex, which by binding to the TRE activates gene transcription, both TH and its receptor, TR, are thought to be important for its expression (317–319). Furthermore, due to

the fact that CaMKIV during development of the nervous system is known to trigger expression of BDNF(320), it is likely that hypothyroidism reduces expression as well as BDNF signalling during this period.

3.2.3 ANIMAL STUDIES OF ARSENIC EXPOSURE AND EFFECTS ON TH HOMEOSTASIS

8 animal studies, focusing on effects of arsenic exposure on TH homeostasis, were identified and included among the search results during the literature search(**table 5**). In only 3 of these, neurotoxic effects were investigated(142–144). However, due to the already mentioned recognized importance of TH for brain development, studies not investigating neurotoxic effects of arsenic exposure were included among the search results. In addition, experimental studies using adult animals were included, due to the already mentioned importance of maternal (and, therefore, adult) TH levels for brain development of offspring.

Table 5. Animal studies identified during literature search, focusing on effects of arsenic exposure on TH homeostasis

Author	TH Biomarkers	Tissue/ matrix	Species	Exposure period	Exposure level and arsenic species	Main findings
Guan et al. (2016)	fT3, fT4, retinoid X receptor (RXR) and TR	Serum and cerebellum	Mouse	60 days	Arsenic trioxide at 1, 2 and 4 mg/L	No significant effect on levels of T3 and T4. Learning and memory impairments, significantly reduced expression of CaMKIV protein and a significantly reduced expression of the thyroid receptor gene β (TR β) and the TR β1 protein in the cerebellum of exposed mice. Shrinkage and loss of Purkinje cells
Guan et al. (2017)	fT3, fT4, and TR	Serum and cerebrum	Mouse	60 days	Arsenic trioxide at 4 µg/L	No significant effect on levels of T3 and T4. Learning and memory impairments and significantly reduced expression of the Trβ gene and of the TRβ1 protein. Morphological changes to the hippocampus
Ahmed and El- Gareib (2019)	fT3, fT4, TSH and thyroid gland	Serum and thyroid gland	Rat	Gestational, GD1-20	Arsenic trioxide at 5 or 10 mg/kg body weight	Reduced levels of T3 and T4, increased TSH. Increased apoptosis, inflammation and oxidative stress. Neurodegenerative changes to the cerebrum, reduced levels of insulin growth factor-I (IGF-1), IGF-II and fetal serum growth hormone. Reduced fetal body- and brain weight
Mohanta et al. (2014)	fT3 and fT4	Serum	Pig	11 weeks	Arsenic trioxide and sodium arsenite at 50µg/L	Significantly reduced levels of T3 and T4.
Sun et al. (2017)	fT4, TRα, TRβ, TSH, TRH, DIO1 and 2	Plasma, brain and thyroid gland	Mouse	8 weeks	Sodium arsenite and sodium arsenate at 10 or 100 µg/L	Damage to the thyroid gland, increase in T4 levels as well as altered transcription of genes in the hypothalamic-pituitary-thyroid (HPT) axis.

Table 5. (continued)

Author	TH Biomarkers	Tissue/ matrix	Species	Exposure period	Exposure level and arsenic species	Main findings
Sun et al. (2015)	fT4, TRα, TRβ, TSH, TRH, DIO1 and 2, TSHβ, TG and TTR	Whole tissue homogenate (T4), brain and liver (HPT-axis genes)	Zebra-fish	48 hours	Sodium arsenite at 0-4.2 mg/L	Altered secretion, transport and conversion of TH. Significantly increased T4 levels, as well as altered transcription of genes in the HPT axis. Decreased mRNA transcription of TRα and no changes in expression of TRβ.
Bashandy et al. (2016)	fT3 and fT4	Plasma	Rat	8 weeks	Sodium arsenite at 6.3 mg/kg body weight	Significantly deceased levels of T3 and T4
Sun et al. (2016)	fT4, TRα and TRβ,	Whole tissue homogenate	Bighead carp larva	48 hours	Sodium arsenite and sodium arsenate at 0-150 µg/L	Increase in T4 levels and reduced mRNA transcription of the thyroid receptors TRα and Trβ.

In three of the studies, Sun et al.(2015), Sun et al. (2016) and Sun et al. (2017) reported increased levels of T4 in zebrafish, bighead carp larva and mouse, respectively. Furthermore, in these studies, it was suggested that these effects were species-specific(147,148,321). As a contrast, Bashandy et al. (2016) Mohanta et al. (2014) and Ahmed and El-Gareib (2019) reported significantly reduced levels of both T4 and T3 in pig and rat, respectively(144–146). The reduced levels of THs reported in these studies, is consistent with AOPs 42 and 54, that are both relevant for developmental neurotoxicity(138,139). Furthermore, in the following, the findings in all animal studies will be described, and other consistencies (apart from reduced levels of THs) with AOPs will be specifically noted.

3.2.3.1 Effects on CaMKIV, TRs and THs

In a study by Guan et al. (2016), no significant effects on arsenic exposure on levels of T3 and T4 in mice exposed to arsenic was reported(324). However, Guan et al. (2016) observed learning and memory impairments, significantly reduced expression of CaMKIV protein and a significantly reduced expression of the thyroid receptor gene β (TR β) and the TR β1 protein in the cerebellum of exposed mice(324). The findings by Guan et al. (2016) are consistent with results from a previous study by Wang et al. (2009). Here, also, arsenic caused reduced expression of CaMKIV in mouse cerebellar tissue and learning and memory impairments was observed(322). In addition, Guan et al. (2016) reported shrinkage and

oedema of Purkinje cells and even loss of Purkinje cells, depending on exposure level(142). In this context, it may be relevant that exposure to environmentally relevant concentrations of arsenic, previously has been shown to damage cerebellar Purkinje cells as well as neurons in the cerebral cortex(323). Furthermore, although Guan et al. (2016) reported no significant reduction in levels of T3 and T4, the significantly reduced expression of the TR β gene and TR β1 protein might have disrupted TH-mediated downstream signalling, which, in turn, might have caused neurodegeneration and learning and memory impairments. It is well known that the cerebellum is essential for motor function(324), and it has long been thought of as a control centre for motor learning, planning of movement and coordination(325). In addition to its well established role for motor function, recently, both human and animal studies indicate that the cerebellum also is crucially important for learning, memory, emotion, cognition and perception(326–329). This is due to neuronal projections from the cerebellum to other brain regions, especially the frontal cortex(325). Through these projections, the cerebellum also is important for for example attention and addiction behaviour(330). Because all connections from the cerebellum to other brain regions converge on Purkinje cells as a central hub, it is likely that any damage to the Purkinje cells, also will affect these projections(325). T3 is indispensable for development of the cerebellum, in particular, for the formation of axodendritic connections between granular neurons and Purkinje cells(330). In addition, TH is important for the migration of precursor cells to the cerebellum as well as to the hippocampus and cerebellar cortex, and these brain organs show the most pronounced anatomical abnormalities due to hypothyroidism (325).

In another study identified during the literature search and similar to Guan et al. (2016), Guan et al. (2017) reported no significant effects on serum T3 and T4 levels, after exposing mice to arsenic(143). Also similar to Guan et al. (2016), exposure was shown to cause a significant reduction in the expression of the Trβ gene and of the TRβ1 protein in the brain of exposed mice. Furthermore, similar to Guan et al. (2016), Guan et al. (2017) reported learning and memory impairments in exposed mice. In addition, arsenic caused morphological changes to hippocampal neuronal cells in this group of animals(143). Guan et al. (2017) observed that co-administration of taurine + arsenic upregulated expression of Trβ1 and that taurine + arsenic ameliorated the effects on morphological changes to the hippocampus as well as the effects on learning and memory seen in the group of animals

exposed to arsenic alone(143). Due to the antioxidant properties of taurine(235–237), Guan et al. (2017) suggested that the effects observed as a result of arsenic exposure were mediated by oxidative stress and that taurine attenuated these effects by direct or indirect mechanisms(143).

Consistencies with AOPs identified for developmental neurotoxicity

The learning and memory impairments reported by Guan et al. (2016) is consistent with AOPs 13 and 54, who both have learning and memory impairment as an AO (AO341) and who both are relevant for developmental neurotoxicity(137,138)(**figure 7**). Furthermore, morphological changes to the hippocampus and learning and memory impairments reported by Guan et al. (2017) is consistent with AOP42, which includes altered hippocampal anatomy as a KE (KE757) and which describes how TPO inhibition as a MIE, causes cognitive impairment as an AO(341)(**figure 8**). In addition, learning and memory impairments reported by Guan et al. (2017) are consistent with AOPs 13 and 54(151,153) (**figure 7**).

3.2.3.2 Effects on THs: Suggested associations with insulin growth factor-I (IGF-1), IGF-II and fetal serum growth hormone (GH)

In another study identified during the literature search, Ahmed and El-Gareib (2019) found that gestational arsenic exposure caused hypothyroidism at gestational day 20 (GD 20) in both rat dams and fetuses after arsenic exposure from GD1-GD20(144). Furthermore, significantly elevated lipid peroxidation, along with significantly increased levels of NO and H_2O_2 was measured in the fetal cerebrum, indicating increased oxidative stress. In addition, signs of increased apoptosis and inflammation were seen in a significant upregulation of mRNA transcription of NF-κB, cyclooxygenase 2 (COX-2), iNOS, BAX and caspase-3 in fetal cerebrum(144). Furthermore, Ahmed and El-Gareib (2019) reported significantly reduced levels of IGF-1, IGF-II and fetal serum growth hormone (GH) in exposed rats and suggested that these effects were associated with maternofetal hypothyroidism. In this context, was maternofetal hypothyroidism suggested by Ahmed and El-Gareib (2019) to be associated with reduced fetal body- and brain weight, that also was observed. As an alternative, Ahmed and El-Gareib (2019) proposed that the adverse effects on fetal growth might have been mediated by the increased levels of ROS in exposed animals or by reduced appetite of the dams due to arsenic exposure(144). Furthermore, Ahmed and El-Gareib

(2019) reported morphological changes to the fetal thyroid gland like for example oedema, degeneration and flattening of colloid vacuoles. In addition, gestational exposure caused visible, histological changes to the fetal cerebrum, as seen by pericellular oedema, congestion of blood vessels, hyperplasia and vacuolar degeneration(144). Ahmed and El-Gareib (2019) suggested that also these neurodegenerative changes could have been caused by maternofetal hypothyroidism. Alternatively, they found it likely that neurodegenerative changes could be due to increased oxidative stress, increased mRNA transcription of proteins and enzymes associated with inflammation and oxidative stress or due to decreased mRNA transcription of PPARγ and Nrf2(144).

Consistencies with AOPs identified for developmental neurotoxicity

The hypothyroidism, neurodegenerative changes and reduced brain weight reported by Ahmed and El-Gareib (2019) might be consistent with AOP54 (**figure 7**), which has learning and memory impairments as an AO and which includes decreased TH synthesis (KE277), decrease of synaptogenesis (KE385) and decrease of neuronal network function (KE386) as key events(138).

3.2.3.3 Limitations of animal studies

Limitations of the animal studies identified are, for example, that concentrations of arsenic used varied strongly and that different arsenic species were used. Another limitation is that those by Ahmed and El-Gareib (2019), Guan et al. (2016) and Guan et al. (2017) were the only ones identified that examined neurotoxic effects of exposure. Furthermore, Sun et al. (2015), Sun et al. (2016) and (Sun et al. (2017) reported, as mentioned, increased levels of T4 in zebrafish, bighead carp larva and mouse, respectively. As a contrast, in other animal studies (Ahmed and El-Gareib (2019), Mohanta et al. (2014) and Bashandy et al. (2016)), decreases in both T4 and T3 were reported. These ambiguities might be due to species-dependent differences in how TH homeostasis is affected by arsenic exposure and other toxic insults. Indeed, in different species, different receptors and enzymes can influence function of THs. In addition, species differences may exist in the different functions of THs across various organisms(310). However, despite the fact that some species differences exist(332), a strong weight of evidence supports the use of THs as biomarkers across vertebrate species (333).

Ahmed and El-Gareib (2019) and Bashandy et al. (2016) used rats in their experiments. Similarly, most animal studies that have examined effects on TH homeostasis, caused by various factors, have used rats. The main serum binding protein for T4 in humans is thyroxine binding globulin (TGB), whereas in rat serum, transthyretin (TTR) is the main iodothyronine binding protein. Furthermore, binding affinity of TTR is weaker compared to TGB. In addition, species-dependent differences in protein binding affinity for THs is believed to influence T4's serum half-life: Whereas T4 has a half-life of 5-9 days in humans, in rats, it has a half-life of 12-24 hours(334). These and other species differences might translate into differences in quantitative dose-response relationships and regulatory feedback mechanism. Despite these differences, however, is reduced serum TH believed to be a KE that is reliably linked to downstream adverse effects(333).

3.2.4 HUMAN STUDIES OF ARSENIC EXPOSURE AND EFFECTS ON TH HOMEOSTASIS

13 human studies were identified during the literature search (**table 6**), focusing on effects of arsenic exposure on TH. In the majority of these, decreases in either T3 or T4 and decreases in the T3/T4 ratio, as well as increases in TSH were observed. Thus, the majority of these studies are consistent with AOPs 42 and 54(138,139).

Table 6. Human studies identified during the literature search, focusing on effects of As on TH

Author	Study type	Number, age and gender of participants	TH bio-markers	Blood and/ or urinary As levels	Main findings	Adjustment factors
Khan et al. (2019)	Cross-sectional	32, male and female, 15-17 years	TSH, tT3 fT4, TPOAb	Low-level lifetime exposure group, urinary (mean ± SD): 66.51 ± 52.27 µg/g creatinine High level lifetime exposure group, urinary (mean ± SD): 328.36 ± 220.47 µg/g creatinine	No significant associations between arsenic exposure and TH parameters. Significant association between neurobehavioural performance and thyroperoxidase antibodies (TPOAb)	Blood pressure, BMI, age, education level
Jain (2016)	Cross-sectional	4126, male and female, 60 ± 12 years (mean ± SD)	TSH, TGN, fT3, tT4, fT3, tT3	Urinary total arsenic: <5.39 (first tertile)->12.3 (third tertile), urinary dimethyl-arsinic acid (UDMA): < 2.61 µg/L (first tertile)- > 5.14 µg/L (third tertile), urinary arsenic adjusted for arsenobetaine (UAAS): < 4.35 µg/L(first tertile)- > 8.9 µg/L (third tertile)	Urinary levels of dimethylarsinate (DMA) were significantly and negatively associated with fT3, tT3 and tT4. Iodine deficient males: Positive and significant association between urinary arsenic levels and TSH and significant, negative association between urinary arsenic and tT4. Iodine-replete males: urinary arsenic were significantly and negatively associated with fT4. Iodine-replete females: Significant, negative association between urinary arsenic and TSH and thyroglobulin (TGN), respectively	Age, gender, race, ethnicity, smoking status, iodine status, BMI, C-reactive protein (CRP), fasting time before blood sampling, urinary creatinine, female estrogen use and additional drug use
Liang et al. (2020)	Birth cohort study	2089 mother-newborn pairs	TSH fT4	Geometric mean of maternal blood arsenic across 3 trimesters: 1.74 µg/L, 1.81 µg/L and 1.99 µg/L. Cord serum: 1.9 µg/L.	Cord serum fT4 levels in newborns were negatively and significantly associated with matenal arsenic exposure during 1. and 2. trimester and average maternal exposure. No significant associations between maternal arsenic exposure and maternal TH parameters.	Gestational age, education level, BMI, gestational hypertension and diabetes, co-exposure to other metals
Guo et al. (2018)	Birth cohort study	915 pregnant women, aged 30.19 years ± 4.09 (mean ± SD)	fT3, tT3, fT4, tT4	Geometric mean level in blood during 20-28 weeks of gestation: 3.88µg/L	Significant, negative association between levels of tT3 and fT3 and arsenic exposure	Gestational age, education level, BMI, smoking status, alcohol intake, gestational hypertension and diabetes, co-exposure to other metals
Sun et al. (2019)	Birth cohort study	675 pregnant women, mean age 29 years	TSH, fT4, fT3	Mean urinary level: 20.03 µg/L	Increased levels of fT4, as well as decreases in both fT3 and the fT3/fT4 ratio.	Gestational age, education level, BMI, smoking status, alcohol intake, gestational hypertension and diabetes, co-exposure to other metals

Table 6. (continued)

Author	Study type	Number, age and gender of participants	TH biomarkers	Blood and/ or urinary As levels	Main findings	Adjustment factors
Molin et al. (2017)	Randomized control trial	38 male and female adults aged 20-40 years	fT3, fT4, TSH	Mean value in blood: 14.2 µg/L	Significant increase in TSH compared to control group	None mentioned
Ciarrocca et al. (2012)	Cross-sectional	313 males	fT3, fT4, TSH, Tg	Mean urinary levels: 10.4 µg/L and 5.2 µg/L	Arsenic exposure was positively associated with TSH and thyroglobulin (Tg) and negatively associated with fT3 and fT4	Age, BMI, smoking status, alcohol intake, diet.
Jurdziak et al. (2018)	Cross-sectional	102 male and female, mean age 45.08 ± 9.87 years	TSH	Mean urinary level: 14.31 µg/g creatinine	No association found between arsenic exposure and TSH. In participants exposed to cadmium, lead and arsenic, higher blood levels of cadmium appeared to increase the risk of disruption of TH homeostasis	Age, BMI, smoking status, hypertension, diabetes, coronary artery disease, dyslipidaemia.
Gong et al. (2015)	Cohort study	723 male and female 50-80 years	T4 and TSH	Not mentioned	Exposure to ≥ 8 µg/L groundwater arsenic and cumulative arsenic exposure multiplied by number of years living in the same area "were significant predictors of hypothyroidism"	Diagnosed hypothyroidism, obesity, iodine status, age, income, health insurance.
Rivera-Núñez et al. (2021)	Birth cohort study	815 pregnant women 27 ± 5 years (mean ± SD)	fT4, T3, T4 and TSH	Mean urinary level: 11.3 ng/ml Mean value in blood: 0.33 ng/ml	No associations found between blood arsenic concentrations and TH	Education level, BMI, smoking status, alcohol intake
Kim et al. (2022)	Cross-sectional	4387 male and female, mean age males: 48.9 , mean age females: 48.8	tT3, tT4, fT3, fT4 and TSH	Mean urinary levels, males: 5.65 (0.11 SD), females: 6.04 (0.14 SD)	Negative association between arsenic exposure and tT3 and tT4	Age, race, BMI, Serum cotinine (ng/mL), smoking status, education, menopausal status, thyroid disease, hormone therapy
Wang et al. (2020)	Prospective cohort study	646 mother-infant pairs, ≥ 18 years	fT4, TSH	Maternal urinary concentrations, 1. trimester: 12.65, 20.76, 35.51 and 82.76 (25th-95th percentile). 3.trimester: 9.4, 14.96, 24.96 and 59.24 (25th-95th percentile)	No significant association between arsenic exposure and TH levels	Maternal age, gestational age, education, exposure to passive smoking, infant gender
Hu et al. (2021)	Cross-sectional	329 male and female, aged 18-83	fT3,fT4, TSH	Urinary concentrations: 0.94, 2.09, 22.43 and 43.75 (25th to 95th percentile)	Significant, negative association between urinary arsenic and fT3	Age, gender, BMI, education, income, sleeping time, smoking status, alcohol intake, physical activity, fasting blood glucose (mmol/L), total cholesterol (mmol/L), triglycerides (mmol/L), urinary iodine/creatinine ratio (µg/g)

3.2.4.1 Studies focusing on effects on TH in newborns

Wang et al. (2020) found no significant associations between arsenic exposure and TH parameters in mothers and infants, respectively(335). In another study, Liang et al. (2020) compared maternal and cord serum arsenic concentrations and found no associations between maternal arsenic exposure and effects on maternal TH. As a contrast, a negative association was found between maternal arsenic levels and cord serum fT4 levels in the first and second trimester(336). Furthermore, a positive association was found between neonatal TSH and cord serum arsenic levels. Such an increase in TSH is likely a compensatory feedback mechanism as a response to reduced levels of T3 or T4 caused by arsenic exposure(337,338). The fact that TH parameters were affected in newborns, while the maternal TH parameters remained unaffected, might indicate that thyrotoxicity can occur in newborns at much lover concentrations of arsenic(336). A likely explanation for this increased vulnerability is the still immature BBB during fetal development(9). Added to this comes the fact that arsenic, as mentioned, easily crosses the placental barrier(15–18). This increases the vulnerability of, for example, the brain. The pituitary, which is a brain structure forming part of the HPT-axis and important for thyroid hormone homeostasis, also appears to be particularly vulnerable to the effects of arsenic exposure: One study by Sánchez-Pena et al (2010), for example, has found accumulation of arsenic in all brain regions of mice, and particularly, in the pituitary(105).

3.2.4.2 Effects on TPO, thyroperoxidase antibodies (TPOAb) and neurobehaviour

In the study by Khan et al. (2019) identified during the literature search, there was found a significantly negative association between arsenic exposure and neurobehavioural performance, as shown by learning and memory impairments, as well as positive associations between arsenic exposure and levels of thyroperoxidase antibodies (TPOAb) and TSH, respectively, among Bangladeshi adolescents(242). Malnutrition, which can exacerbate the adverse effects of environmental toxicants is, as already mentioned, a common confounder in studies that have reported adverse neurobehavioural effects of arsenic exposure(25), and in the study by Khan et al. (2019), the participating adolescents lived under marginal socio-economic conditions, which, in itself, may have been associated

with malnutrition(242). In addition, TH parameters may, for example, be affected by iodine, selenium and iron status, and deficiencies in all these elements might occur in cases of malnutrition(317,318). Thus, deficiencies in one or more of these micronutrients are likely confounders in the study by Khan et al. (2019). In this context, it is possible that arsenic, as previously mentioned, depletes the body's supply of selenium(341), which, in turn, might affect TH parameters. Furthermore, Khan et al. (2019) found, as mentioned, significant, positive associations between levels of TPOAb and arsenic exposure. Similar, positive associations have been reported between TPOAb and exposure to for example polyhalogenated biphenyls and various metals(342–344). In addition, increased levels TPOAb that were found by Khan et al. (2019) is a possible indication of autoimmune thyroid disease, which may be a consequence of exposure to environmental toxicants. By perturbing the function of the TPO enzyme, TPOAb might reduce levels of TH(342). Another possibility is that arsenic's inhibition of TPO is mediated, not by antibodies, but by direct inhibition its activity. Such inhibition has previously been reported in studies not identified during the literature search, by Palazzolo and Ely (2015) and Palazzolo and Jansen (2008)(345,346), and it has been suggested that As_2O_3 inhibits TPO by binding to its free sulfhydryl groups(346). TPO is a large enzyme containing more than 900 amino acids, with a molecular weight of about 100 kDa(347). Because of its size and three dimensional structure, it is highly probable that it contains sulfhydryl groups that are accessible for binding to arsenic compounds like for example As_2O_3. A likely consequence of this binding is configurational changes to the active site of TPO. These changes to the configuration, in turn, may inhibit iodine oxidation, which is required for thyroid hormone synthesis(346).

Consistencies with AOPs identified for developmental neurotoxicity

The significantly increased levels of TSH, TPOAb and the learning and memory impairments reported by Khan et al. (2019) are consistent with AOP54(138) (**Figure 7**). Furthermore, the associations reported between arsenic exposure, increased levels of TSH, TPOAb and impaired cognitive performance is consistent with AOP42 (**figure 8)**, which describes how TPO inhibition (MIE279) leads to decreased cognitive function as an AO (AO402)(139)

.

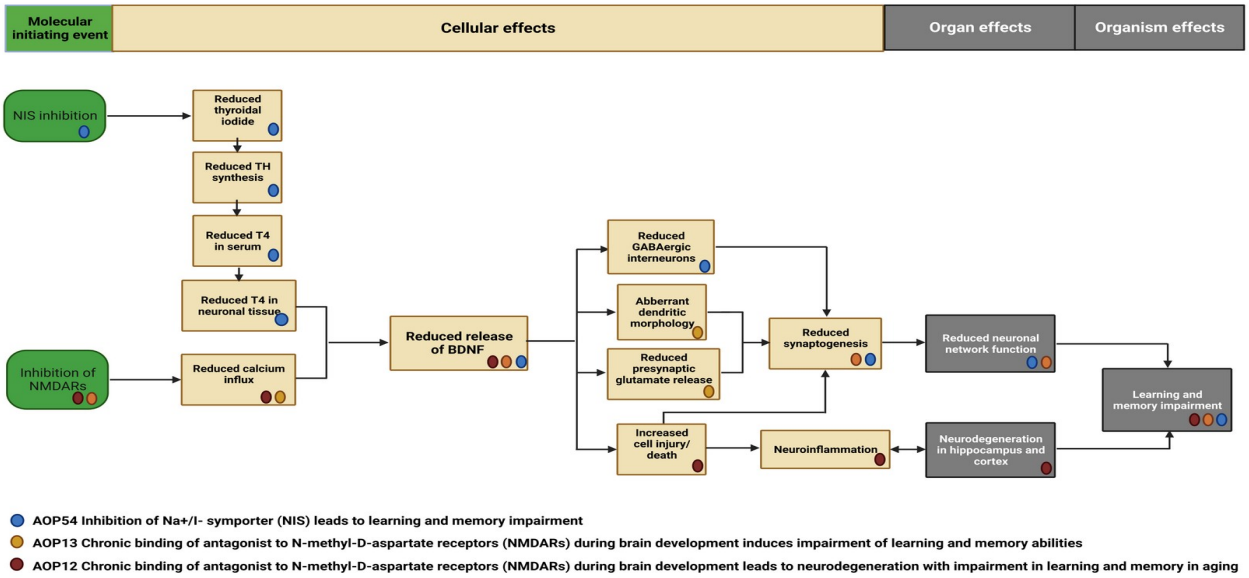

Figure 7) Schematic representation of AOPs 12, 13 and 54. The reduced hippocampal neurogenesis reported by Tyler et al. (2014) might be consistent with KE341 ("impairment, learning and memory"), which is involved in AOP 13 and 54. The association that was found by Sun et al. (2015) between arsenic exposure, reduced expression of BDNF and impaired object recognition LTM, respectively, is consistent with KE341 and AOPs 13 and 54. Pandey et al. (2017) reported that arsenic exposure caused neuronal loss, a decrease in neuronal perimeter as well as a decrease in dendritic length and number of dendritic branches(197). These findings, as well as the reduced expression of BDNF reported by Pandey et al. (2017), are consistent with AOP13. Furthermore, the learning and memory impairments reported by Guan et al. (2016) is consistent with AOPs 13 and 54. The hypothyroidism, neurodegenerative changes and reduced brain weight reported by Ahmed and El-Gareib (2019) might be consistent with AOP54, which includes decreased TH synthesis (KE277), decrease of synaptogenesis (KE385) and decrease of neuronal network function (KE386) as key events(138). The significantly increased levels of TSH, TPOAb and the learning and memory impairments reported by Khan et al. (2019) are consistent with AOP54

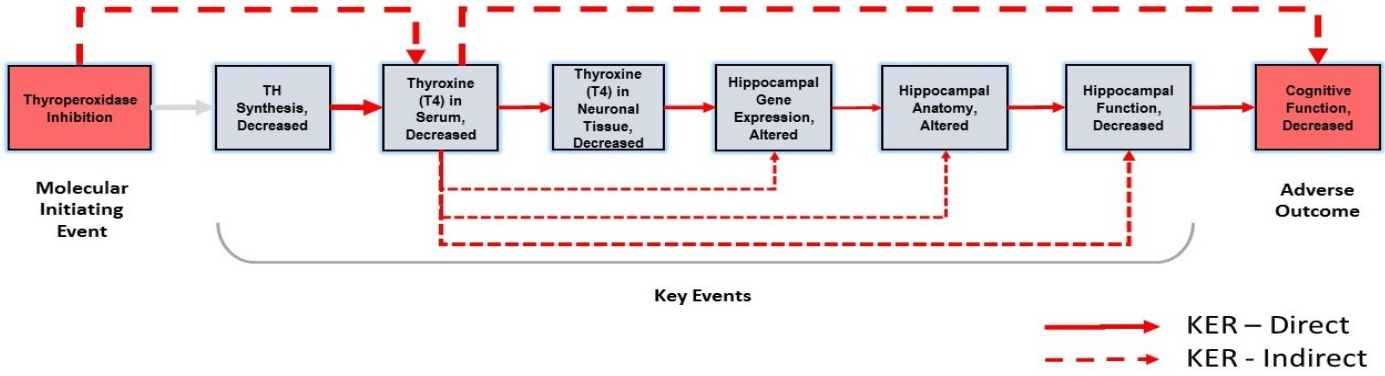

Figure 8) AOP54. It is is likely that the reduced hippocampal neurogenesis reported by Tyler et al. (2014) is consistent with AOP42, which includes altered hippocampal anatomy (KE757), altered hippocampal physiology (KE758) and decreased cognitive function (AO402) as KEs and AO, respectively. Neuronal loss, decrease in neuronal perimeter as well as decrease in dendritic length and number of dendritic branches reported by Pandey et al. (2017)(197) may be consistent with AOP54. Furthermore, morphological changes to the hippocampus and learning and memory impairments reported by Guan et al. (2017) is consistent with AOP42. The associations reported between arsenic exposure, increased levels of TSH, TPOAb and impaired cognitive performance reported by Khan et al. (2019) is consistent with AOP42.

3.2.4.3 Effects on the fT4/fT3 or fT3/fT4 ratio

Sun et al. (2019) found increased levels of fT4, as well as decreases in fT3 and decreases in the fT3/fT4 ratio among 675 pregnant Chinese women exposed to several metals, including arsenic(348). The fT3/fT4 ratio is used as a measure of how efficiently the deioidinase enzymes (DIO1 and DIO2) convert T4 to T3(348). Furthermore, in a study by Molin et al. (2017), there was observed a significant increase in the **fT4/fT3** ratio (equivalent to a *decrease* in the **fT3/fT4** ratio) in humans ingesting seafoods.

Likewise, in another study (not identified during the literature search) by Meltzer et al (2002), blood arsenic concentrations were significantly associated with increases in the fT4/fT3 ratio among volunteers ingesting fish at least three times weekly for 15 weeks(349). The results from the studies by Sun et al. (2019), Molin et al. (2017) and Meltzer et al (2002) suggest that both organic as well as inorganic arsenic species negatively affect the function of DIO1 and DIO2 enzymes. In the majority of the other human studies identified during the literature search, arsenic exposure was shown to be associated with reduced levels of T3. Possibly, these effects were mediated by reduced function of the DIO1 and DIO2 enzymes.

In this context, it is relevant to mention two animal studies identified during the literature search, by Sun et al. (2015) and Sun et al. (2017), who reported reduced mRNA transcription of DIO1 and DIO2 in zebrafish and mice, respectively, as a result of arsenic exposure(147,148). Furthermore, this is consistent with results from a study by Davey et al. (2008) where low-dose arsenic exposure caused a significant downregulation of the DIO1 gene *in vitro (350)*. Because the deiodinase enzymes are dependent on selenium for their function(291) and because arsenic may bind to and increase excretion of selenium(341), it is possible that arsenic's effect on the function of DIO1 and DIO2 is mediated by its effect on the body's selenium levels(148).

3.2.4.4 Limitations of human studies

Arsenic may disrupt mechanisms of action of THs in some tissues, despite normal blood TH parameters under various physiological conditions(53,351). Thus, in some cases, blood THs might not be a good predictor of neurotoxic effects of arsenic exposure mediated by disruption of TH homeostasis(242). Furthermore, in the context of neonatal TH status,

normal TH-levels measured in a newborn do not rule out that TH levels may have normalised at birth, after a preceding period of hypothroidism during gestation. Thus, a normal TH status does not preclude neurodevelopmental deficits due to hypothyroidism during gestation(277).

One clear limitation with the human studies identified during the literature search was that, with the exception of the one by Khan et al. (2019), none investigated possible associations between arsenic exposure,TH levels and neurobehavioural performance, respectively. Another, is that the majority of them were cross-sectional (Jain (2015), Guo et al. (2018), Khan et al. (2020), Ciarrocca et al. (2012), Jurdziak et al. (2018), Kim et al. (2022) and Hu et al. (2021)), precluding the possibility of linking causes to effects.

Yet another limitation is that some of them had small sample sizes (Khan et al. (2020), Molin et al. (2017), Wang et al. (2020)). Furthermore, those by Khan et al. (2019), Liang et al. (2020) and Wang et al. (2020) were the only ones where participants were newborns and adolescents.

Micronutrient deficiency was mentioned previously as a potential confounder in human studies assessing neurotoxic effects of arsenic exposure. Such deficiencies may also affect TH homeostasis in humans(352), and in the human studies of arsenic's effect on TH identified during the literature search, it was not consistently adjusted for, for example, iodine status, which is a micronutrient that can affect TH homeostasis(358). As such, iodine status was not used as a covariate in the studies by Sun et al. (2019), Guo et al. (2018), Molin et al. (2017), Rivera-Núñez et al. (2021), Jurdziak et al. (2018) and Wang et al. (2020). Furthermore, many other covariates were used, who also were inconsistently adjusted for.

Another limitation with the human studies is that, in none of them, arsenic speciation was conducted. In the studies by Liang et al. (2020), Guo et al. (2018), Rivera-Núñez et al. (2021), Kim et al. (2022), Hu et al. (2021), Wang et al. (2020) and Sun et al. (2019), effects on TH parameters of multiple metals found in blood of participants was examined. In these, linear regression models and quantile-based g-computation (QGC) (Kim et al. (2022)) were used to examine effect of arsenic alone on TH(335,336,348,353–355). These methods can only provide an estimation of the isolated effect of arsenic on TH parameters. However,

environmental exposure to multiple metals (as well as several other environmental toxicants potentially affecting TH homeostasis) represents a realistic scenario for humans, compared to single-metal exposure(254). In their environment, humans are exposed to a large number of chemicals; a "toxic cocktail", which is thought to target TH homeostasis(325). Among these compounds are for example bisphenols, phthalates and polybrominated diphenyl ethers (PBDEs), and, with the exception of Hu et al. (2021), who examined effects of PBDEs and metal mixtures on thyroid function, no other human studies adjusted for this background exposure(325).

Another possible limitation with the studies, is that chronic disease conditions affecting the cardiovascular system as well as liver and kidney function, also may affect TH homeostasis(356–362), and data concerning these covariates were not consistently collected. Furthermore, the studies differed in whether blood or urine was used to measure arsenic exposure: Whereas Molin et al. (2017) and Rivera-Núñez et al. (2020) measured arsenic concentrations in blood and urine and Guo et al. (2018) and Liang et al. (2020) in blood, the remainder measured urinary arsenic concentrations. The advantages and disadvantages of urinary and blood measurements, respectively, are discussed above. Likewise, the increased accuracy that can be achieved by combining blood and urine measurements was mentioned. Further limitations with the studies are that the various potential sources of arsenic exposure were not investigated. Such an investigation might have made it possible to assess the impact of exposure to inorganic arsenic and organoarsenicals, respectively, on TH homeostasis. The study by Molin et al. (2017)(363) was the only one that specifically investigated effects of exposure to organoarsenicals on TH parameters.

Finally, procedures for measurements of TH were not uniform and standardized and different TH parameters were used. Due to the variations and inconsistencies mentioned above, it appears difficult to draw firm conclusions about associations between arsenic exposure and disruption of TH homeostasis in humans. In the case of children and adolescents, it is especially difficult to draw firm conclusions, due to the small number of studies that were identified, focusing on these age groups.

3.2.4.5 MEASUREMENT OF THs IN HUMANS

Because of their already mentioned pleiotropic effects and importance for development, serum levels of TSH and T4 are measured in newborns as part of routine examinations to

diagnose congenial hypothyroidism(314). T3 and T4 are measureable in free and unbound form (fT3 and fT4) or as levels of total T3 and T4 (bound + unbound, tT3 and tT4)). ELISA or radioimmunoassay (RIA) kits are in most cases used for measurements(333). However, recently, use of high performance liquid chromatography (HPLC) as well as mass spectroscopy has become more common (333). These methods enable direct measurements of THs. As a contrast, RIA and ELISA measure THs indirectly, and RIA is considered to yield the most repeatable and reproducible results(333).

4. CONCLUSIONS AND FURTHER PERSPECTIVES

Among animal studies that used BDNF as a biomarker, several consistencies between main findings and relevant AOPs were found, which strengthens the relevance of BDNF as an effect biomarker in epidemiological studies of arsenic exposure. Likewise, for TH, several consistencies between AOPs relevant for developmental neurotoxicity and main findings in the selected and identified animal studies were found. One of the main strengths of BDNF as a potential effect biomarker in future, human studies of arsenic exposure is that it has previously been suggested as an effect biomarkers in human biomonitoring studies examining effects of exposure to other environmental toxicants on BDNF(140). One of the main limitations with its future use, however, is the small number of human studies of arsenic exposure, where BDNF has been implemented as a biomarker. Conversely, a comparably large number of human studies exist, where negative associations between arsenic exposure and THs have been found. However, as opposed to BDNF, which has been used as an effect biomarker in several experimental studies of neurodegenerative and neurobehavioural effects, comparably few animal and in vitro studies have investigated associations between arsenic exposure, effects on THs and neurodegenerative or neurobehavioural effects.

Weaknesses identified in human studies of associations between arsenic exposure and TH dysregulation included inconsistent adjustment for covariates and that procedures for measurements of TH were not uniform and standardized. In addition, different TH parameters were used. Furthermore, a large number of the human studies were cross-sectional, which precludes the possibility of linking causes to effects. Both BDNF and

TH are together included in AOPs describing developmental neurotoxicity (AOPs 13 and 54), and it is likely that an increased sensitivity and specificity can be achieved if they are being used in combination as effect biomarkers. However, due to the above mentioned limitations, no certain associations between arsenic exposure and effects on TH disruption in humans could be found. This is also true for associations between arsenic exposure and effects on BDNF, due to the small number of human studies that have used BDNF as a biomarker for this type of exposure. In addition, in the animal studies, brain tissue was exclusively used for BDNF measurements. Thus, animal studies provide no information about the possibility of using minimally invasive measurements of BDNF.

The HBM4EU initiative is currently assessing effect biomarkers for their use in human biomonitoring of exposure to several groups of environmental toxicants, including arsenic(140). This ongoing work will facilitate the selection of effect biomarkers and, hopefully, it will broaden our understanding of potential adverse effects of these toxicants. In the future, it is required that the methodologies for the measurement of these biomarkers are harmonized. Furthermore, quality control and inter-laboratory comparisons must be improved, which, in turn, will improve replicability and comparability among studies(140). In epidemiological studies, cross-sectional studies who use single measurements need to be replaced by prospective studies, using repeated measurements of effect biomarkers combined with exposure biomarkers(140).

In addition, in the future, epidemiological studies of neurotoxic effects of arsenic exposure as well as epidemiological studies of similar effects of exposure to other environmental toxicants, may need to harmonize their approaches to how the environmental background exposure to the complex mixture of toxicants is adjusted for. This is because these other environmental toxicants, apart from those, whose levels are being measured, may have additive or synergistic effects.

Many knowledge gaps in the understanding of the mechanisms underlying how environmental toxicants affect for example levels of biomarkers, might be overcome by the use of a combination of multi-omics technologies. These approaches enable the analysis of metabolites (metabolomics), proteins (proteomics), global gene characterisation (genomics), genome-wide epigenetic analyses and analysis of mRNA (transcriptomics)(140). Furthermore, possibly, these multi-omics technologies will shed light on the mechanisms

underlying synergistic and additive effects of complex mixtures of toxicants.

One limitation of the AOPs that were identified for developmental neurotoxicity is that none of them focus on AOs relevant for behavioural and mental disorders (140). Whereas the involvement of BDNF in the development of such disorders is well recognized, BDNF is currently only involved as a KE in AOPs 13 and 54, who describe learning and memory impairments as an AO. Possibly, development of AOPs relevant for behavioural and mental disorders will promote future, human studies of possible associations between exposure to neurotoxicants, BDNF and these types of disorders.

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6. APPENDIX

Table 1. An extensive literature search was performed after studies made available in the US National Library of Medicine (NCBI Pubmed) in the period between June the 1st. 2012 and June the 1st. 2022. MeSH® terms and MeSH Supplementary Concepts (**https://www.nlm.nih.gov/mesh**) along with other search terms were used, as shown below. Search terms for As exposure were combined with search terms for neurobehavioural health endpoints

Arsenic exposure	("Arsenic"[Mesh] OR "Arsenates"[Mesh] OR "Arsenites"[Mesh] OR "arsenic acid"[Supplementary Concept] OR "arsenite"[Supplementary Concept] OR "arsenous acid"[Supplementary Concept] OR "monomethylarsonic acid"[Supplementary Concept] OR "dimethylarsinous acid"[Supplementary Concept] OR ("arsenite"[Supplementary Concept] OR "arsenite"[All Fields]) OR ("arsenic acid"[Supplementary Concept] OR "arsenic acid"[All Fields] OR "arsenate"[All Fields]) OR "Arsenious acid"[All Fields] OR iAs[All Fields] OR "Arsenic trioxide"[All Fields] OR AsIII[All Fields] OR "As(III)"[All Fields] OR AsV[All Fields] OR "As(V)"[All Fields] OR MMA[All Fields] OR "dimethylarsinic acid"[All Fields] OR "dimethylarsenic acid"[All Fields] OR "dimethylarsonic acid"[All Fields] OR DMA[All Fields])
Neurobehaviour	("Nervous System"[Mesh] OR "Nervous System Diseases"[Mesh] OR "Neurogenesis"[Mesh] OR "Child Development"[Mesh] OR "Cognitive Dysfunction"[Mesh] OR "Behavior"[Mesh] OR "Behavior and Behavior Mechanisms"[Mesh] OR "Reproductive Behavior"[Mesh] OR "Social Behavior Disorders"[Mesh] OR "Child Behavior Disorders"[Mesh] OR "Adolescent Behavior"[Mesh] OR "Antisocial Personality Disorder"[Mesh] OR "Infant Behavior"[Mesh] OR "Spatial Behavior"[Mesh] OR "Sucking Behavior"[Mesh] OR "Sexual Behavior, Animal"[Mesh] OR "Sexual Behavior"[Mesh] OR "Paternal Behavior"[Mesh] OR "Maternal Behavior"[Mesh] OR "Impulsive Behavior"[Mesh] OR "Feeding Behavior"[Mesh] OR "Exploratory Behavior"[Mesh] OR "Compulsive Behavior"[Mesh] OR "Child Behavior"[Mesh] OR "Behavior, Animal"[Mesh] OR "Mental Disorders"[Mesh] OR “Neurobehavior”[All Fields] OR “Neurodevelopment”[All Fields] OR “Neurology”[All Fields] OR “Autism”[All Fields] OR “Hyperactivity”[All Fields] OR “ASD”[All Fields] OR “ADHD”[All Fields] OR “mental retardation”[All Fields] OR “IQ”[All Fields])