2016•2017
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Prenatal acrylamide exposure and brain-derived neurotrophic factor in the ENVIRONAGE Birth Cohort Study

Promotor :
dr. Janneke HOGERVORST

Copromotor :
Prof. dr. Tim NAWROT

Sanne Verspeek

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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ACKNOWLEDGEMENTS

Six years ago, I started this adventure thinking: “If you’ll never try, you’ll never know”. I always had ambitions and curiosity leading the way for me, but I had never imagined I would be finishing a master thesis in Biomedical Sciences. Studying at Hasselt University these last years have matured me, but my fellow students have also kept me young. Even though combining studies, work, a household and a personal life while traveling to Belgium every day was definitely not always easy, I would do it all over again in a heartbeat.

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Thanks mom and dad, for supporting me all the way and always believing in me. Without you I could never have done it all. Last but not least, thank you Noud, for pushing me when I needed it and believing in me when I didn’t. You made me persevere and I truly thank you for that.

LIST OF ABBREVIATIONS

AA-Hb	acrylamide-hemoglobin
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CYP2E1	cytochrome P450 2E1
DOHaD	Developmental Origins of Health and Disease
EFSA	European Food Safety and Authority
ELISA	enzyme-linked immunosorbent assay
ENVIR*ON*AGE	ENVIRonmental influence *ON* early AGEing
EPHX1	epoxide hydrolase I
GA-Hb	glycidamide-hemoglobin
GSTs	glutathione-S-transferases
GSTT1	glutathione-s-transferase Theta 1
GSTM1	glutathione-s-transferase Mu 1
GSTP1	glutathione-s-transferase Pi 1
HRP	horseradish peroxidase
IQR	interquartile range
LC/MS/MS	liquid chromatography tandem mass spectrometry
NGF	nerve growth factor
NT-3	neurotrophic factor-3
NT-4	neurotrophic factor-4
PAHs	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction
ROS	reactive oxygen species
SD	standard deviation
SNPs	single nucleotide polymorphisms

ABSTRACT

Background – Acrylamide is a probable human carcinogen present in carbohydrate-rich heat-treated foods commonly consumed worldwide. Recently, inverse associations were found between acrylamide exposure and fetal growth. Brain-derived neurotrophic factor (BDNF) influences fetal growth and plays a vital role in the development of the nervous system. A recent study in fetal rats showed decreased BDNF levels after acrylamide exposure.

Objectives – We investigated, for the first time, if there was an inverse association between prenatal acrylamide exposure and cord blood BDNF levels in humans. Additionally, possible effect modification of this association by genetic variants in acrylamide-metabolizing genes was examined.

Methods – BDNF cord plasma levels of 69 mother-newborn pairs from the ENVIR*ON*AGE birth cohort were measured using a commercially available enzyme-linked immunosorbent assay. Cord blood acrylamide hemoglobin (AA-Hb) adducts, a biomarker for internal acrylamide exposure during the last several months of pregnancy, were measured by means of liquid chromatography-tandem mass spectrometry. Genotyping for single nucleotide polymorphisms (SNPs) in *CYP2E1*, *EPHX1* and *GSTP1* was done using the Biotrove OpenArray SNP Genotyping Platform and copy numbers of *GSTM1* and *GSTT1* were determined using quantitative real-time polymerase chain reaction.

Results – Median (IQR) BDNF and AA-Hb levels were 961 (873) pg/ml and 20.1 (17.3) pmol/g hemoglobin respectively. BDNF levels decreased, borderline statistically significant, by 15.4% (95% CI: -29.3 – 1.2%, $p = 0.07$) for every doubling in AA-Hb levels, after adjusting for parity and gestational age. None of the interactions with genetic variants were statistically significant. However, stronger inverse associations between acrylamide and BDNF were seen for newborns with variant alleles of rs915906 and rs2480258 in *CYP2E1*, rs1695 in *GSTP1*, and the homozygous deletion of *GSTM1*.

Conclusion – A borderline statistically significant association was found between prenatal acrylamide exposure and neonatal levels of BDNF. Considering the inverse trend already visible in this small dataset, future studies with a bigger study population are urgently needed.

SAMENVATTING

Achtergrond – Acrylamide is geclassificeerd als een “mogelijk carcinogene stof”, en is aanwezig in koolhydraatrijke verhitte voedingsmiddelen die wereldwijd geconsumeerd worden (bijvoorbeeld chips, frites, koekjes en koffie). Recentelijk zijn er inverse associaties gevonden tussen blootstelling aan acrylamide en foetale groei. Breingerelateerde neurotrofe factor (BDNF) beïnvloedt foetale groei en speelt een belangrijke rol in de ontwikkeling van het centrale zenuwstelsel. Een recente studie in foetale ratten liet verlaagde BDNF concentraties zien na blootstelling aan acrylamide.

Doelstellingen – We hebben, voor het eerst in de mens, onderzocht of er een inverse associatie bestond tussen prenatale blootstelling aan acrylamide en BDNF-concentraties in navelstrengbloed. Daarnaast zijn mogelijke effectmodificaties, door genetische varianten in acrylamide-metaboliserende genen, van deze associatie onderzocht.

Methoden – In navelstrengbloedplasma van 69 moeder-pasgeboreneparen werden BDNF-concentraties gemeten, middels een commercieel beschikbare enzym-gelinkte immunosorbent (ELISA) test. Ook In navelstrengbloed werden acrylamide aan-hemoglobineadducten (AA-Hb), een biomarker voor interne acrylamide blootstelling, gemeten door middel van vloeibare-chromatografie-tandem-massaspectrometrie. Genotypering voor enkel-nucleotide polymorfismen (SNPs) in *CYP2E1*, *EPHX1* en *GSTP1* werd gedaan door middel van de Biotrove OpenArray SNP Genotyping Platform en kopienummers van de *GSTM1*- en *GSTT1*-genen werden bepaald middels kwantitatieve real-time polymerase- kettingreactie.

Resultaten – De medianen (IQR) van de BDNF en AA-Hb adduct concentraties bedroegen 961 (873) pg/ml en 20,1 (17,3) pmol/g Hb, respectievelijk. We zagen een, op de grens van statistisch significante, afname van de BDNFconcentraties van 15,4% (95% CI: -29,3 – 1,2%, p = 0,07) voor een verdubbeling van de AA-Hb concentraties. Er was geen statistisch significante interactie tussen AA-Hb en de genetische varianten. We zagen wel sterkere inverse associaties tussen acrylamide en BDNF voor pasgeborenen met variant allelen van rs915906 en rs2480258 in *CYP2E1*, rs1695 in *GSTP1*, en de homozygote deletie van *GSTM1*.

Conclusie – In deze studie was er een inverse associatie (op de grens van statistische significantie) tussen prenatale acrylamide blootstelling en neonatale BDNF-concentraties. Gezien de inverse trend reeds

1. INTRODUCTION

1.1 Acrylamide

Acrylamide (C_3H_5NO), is a colorless, odorless and water-soluble solid, with a molecular weight of 71.08 gram/mole. Acrylamide is formed by hydration of acrylonitrile and has been used by the chemical industry since the 1950s as a precursor in the formation of polyacrylamides (1). Polyacrylamides are used in wastewater treatment, soil conditioning and in the production of cosmetics, dyes, plastics and papers among others. Another application of acrylamide lies in laboratory research, where acrylamide is used to make gels for electrophoresis and chromatography (1, 2). In the occupational setting, exposure to acrylamide occurs mainly via dermal contact with the monomer, but also by inhalation of vapor and dust during the production of polyacrylamides (3).

1.2 Dietary acrylamide exposure

In 2002, the Swedish National Food Administration was the first to report that acrylamide is present in regularly consumed foods such as French fries, potato chips, cookies and coffee (4). In food, acrylamide is formed by the Maillard reaction (Figure 1.1), which is a reaction between amino acids and reducing sugars, at temperatures higher than 120°C and at conditions of low moisture content and low acidity. The Maillard reaction is responsible for the browning of food and provides flavor.

Since acrylamide is primarily formed by the reaction of asparagine with fructose or glucose, formation occurs in carbohydrate-rich foods that are baked, fried, grilled or toasted (3, 4). In addition to asparagine and fructose or glucose, external factors influence the amount of acrylamide formed in food. Various studies have shown that acrylamide concentrations increase with an increase in heating temperature and time, reaching a maximum when temperature exceeds 190°C, but also with increasing pH and water content (3). It is estimated that dietary acrylamide exposure ranges from 0.3 – 0.8 µg/kg body weight per day for the general public. For children, acrylamide intake is estimated to be 2 to 3 times higher per kilogram body weight than for adults, due to a higher consumption of acrylamide-containing foods in children.

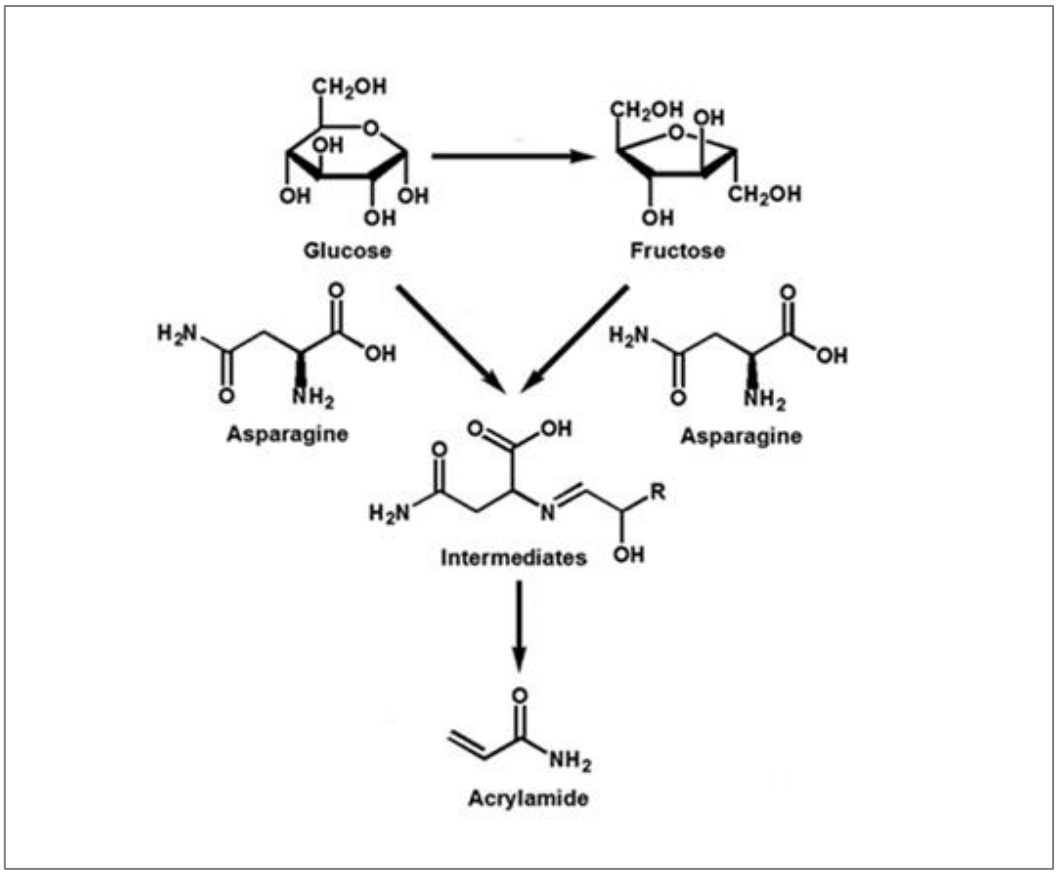

Figure 1.1. Simplified scheme of acrylamide formation via the Maillard reaction (adapted from Zhang *et al*.(5)).

Since nearly everyone is exposed to acrylamide through diet, its presence in food is a serious public health concern (3). Various efforts have already been made by the food industry to decrease levels of acrylamide in food, with methods, such as controlling temperature, heating time and pH of processing and removing free asparagine before processing by using the enzyme asparaginase (6).

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1.3 Acrylamide metabolism

Through ingestion, inhalation or dermal exposure, acrylamide is easily absorbed, distributed throughout the body with the blood, and eliminated in the urine with a half-life of only 3.1 to 3.5 hours. After absorption, acrylamide is partly converted to its epoxide metabolite glycidamide by the cytochrome P450 2E1 (CYP2E1) enzyme via epoxidation. Both compounds can be detoxified through conjugation to glutathione by glutathione-S-transferases (GSTs) and are eliminated in urine as byproducts of mercapturic acids, which is the main route of acrylamide excretion. Furthermore, glycidamide is partly converted to glyceramide by epoxide hydrolase I (EPHX1) and then excreted in urine (4, 7). The main steps in acrylamide metabolism are shown in Figure 1.2.

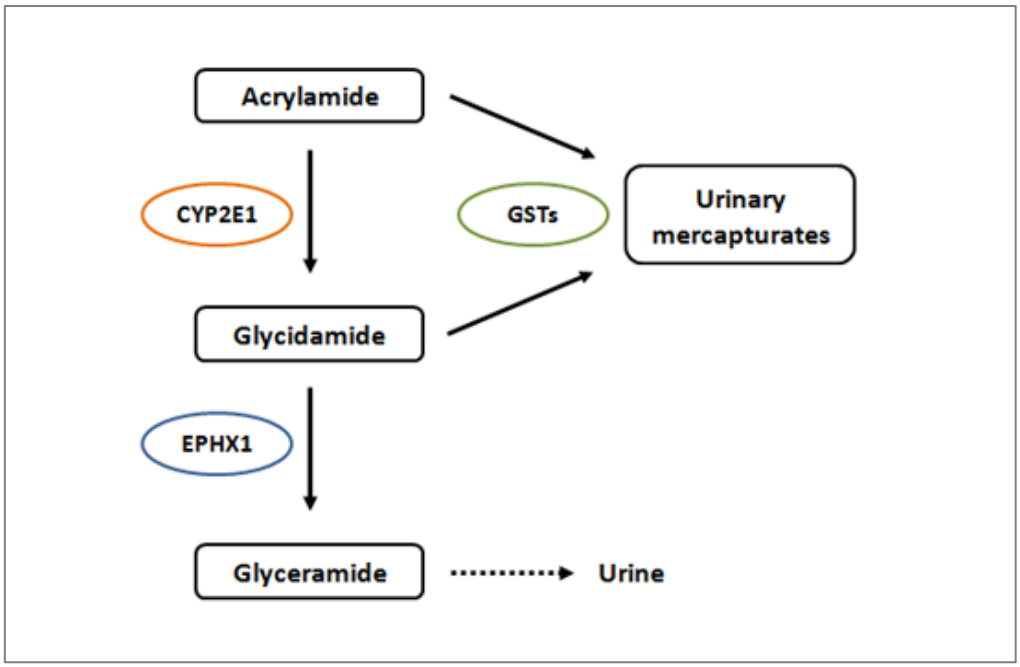

Figure 1.2. Simplified scheme of the metabolic pathway of acrylamide (adapted from Duale *et. al.* (8)). CYP2E1: cytochrome P450 2E1, GSTs: glutathione-S-transferases, EPHX1: epoxide hydrolase 1.

The most frequent type of genetic variation in humans are single nucleotide polymorphisms (SNPs), which are variations in one single nucleotide occurring at specific locations in the genome in more than 1% of the population. Enzymes involved in the activation or detoxification of compounds often have a polymorphic expression, which means that between individuals enzymatic activity can differ (8). Genes encoding the above-mentioned enzymes involved in acrylamide metabolism also have known polymorphisms. Animal studies have shown higher levels of acrylamide adducts in CYP2E1-null mice in comparison to wild-type mice after acrylamide exposure (8). Known genetic variants that alter the activity of GSTs are for instance the homozygous deletions of *GSTM1* and *GSTT1* genes, along with the 105Val allele polymorphism (rs1695) in *GSTP1* (8). EPHX1 enzyme activity is shown to be affected by at least two polymorphisms comprising the substitutions of amino acids which can decrease or increase enzyme activity (8).

Acrylamide and glycidamide are both reactive compounds that form adducts with hemoglobin in red blood cells. These hemoglobin adducts are a representation of the internal concentrations of acrylamide and glycidamide in the circulation over the lifetime of red blood cells, which is approximately 4 months. Therefore, acrylamide and glycidamide hemoglobin (AA- and GA-Hb) adducts are considered to be a biomarker for internal exposure (7, 8). As reviewed by Mandeep *et al*. (7), age and race influence hemoglobin adduct levels as well as smoking status, since acrylamide is also present

in cigarette smoke in high concentrations. The U.S. National Health and Nutrition Examination Survey 2003 – 2004 showed AA- and GA-Hb adduct levels to range between 3 – 910 and 4 – 756 pmol/g hemoglobin, respectively, in adults (7).

1.4 Acrylamide and health

In 1994, acrylamide was classified as a probable human carcinogen (group 2A carcinogen) by the International Agency for Research on Cancer, based on evidence on its carcinogenic potential found in animal studies and the probably genotoxic mechanism of action which is thought to operate in humans as well (1). Since acrylamide is an electrophilic compound, it reacts readily with molecules that contain nucleophilic sites, such as amino and sulfhydryl groups in proteins and amino acids. Glycidamide has the ability to react with nucleophilic sites as well, but to a lesser extent. However, it has been shown that glycidamide is 100 – 1000 times more reactive with DNA than acrylamide. Therefore, acrylamide and/or its metabolite glycidamide have toxic, mutagenic and carcinogenic properties (2).

Experimental studies in rats and mice, orally exposed to high levels of acrylamide, have shown an increased incidence of various tumors such as tumors of the mammary gland, thyroid gland, and central nervous system (4). In the last decade, numerous studies went out to the possibility of acrylamide to cause cancer in humans. Some but not all epidemiological studies reported positive associations between dietary acrylamide intake and an increased risk of postmenopausal endometrial and ovarian cancer, and renal cell cancer (9).

Reproductive and developmental toxicity were seen in experimental animals after exposure to acrylamide, such as disrupted mating, impaired fertility, reduced litter size and reduced fetal growth (3). Recently, several epidemiological studies showed associations between dietary acrylamide exposure and effects on fetal development. Maternal dietary intake of acrylamide was inversely associated with fetal head circumference, birth weight and length (10-12).

The first adverse health effects seen in humans due to acrylamide exposure were effects on the peripheral and central nervous system. Various epidemiological studies reported on the incidence of neurotoxicity in people exposed to acrylamide by occupation. In China, factory workers who were sub-chronically exposed to acrylamide suffered from reversible peripheral neuropathy (ataxia, tingling, weakness and numbness in limbs) (3). More severe symptoms were seen after longer exposure, including cerebellar dysfunction and subsequently neuropathy (3). Reversible peripheral nervous system symptoms were also seen in Swedish tunnel workers, exposed to a grouting agent containing acrylamide. In animal studies, chronic exposure to acrylamide showed similar results regarding

neurotoxicity (2). These observations suggest that acrylamide could have a cumulative effect regarding neurotoxicity. Although the high exposure levels of acrylamide that are seen in the occupational setting are not reached through the diet, it is possible that long-term low exposures to dietary acrylamide have cumulative effects on the human nervous system.

1.5 Neurodevelopmental susceptibility to acrylamide

The fetal period of development is extremely susceptible to perturbations of the intrauterine environment, caused by changes in maternal nutrition and exposures to environmental pollutants among other. A growing body of experimental, clinical and epidemiological research shows that these perturbations during early development increase the risk of developing cardiovascular, metabolic, respiratory and neurological diseases later in life. This concept, originating from the Barker Hypothesis, is called the Developmental Origins of Health and Disease (DOHaD) (13-15).

Until recently, knowledge on fetal exposure to acrylamide through the maternal diet was still limited. Annola *et al.* (16) showed that acrylamide can cross the placental barrier *in vitro*. By measuring AA- and GA-Hb adducts in maternal blood and cord blood, Von Stedingk *et al.* (17), showed that after dietary acrylamide exposure both compounds pass the placenta and that the fetus receives approximately the same dose as the mother.

An experimental animal study recently showed that dietary acrylamide exposure (5 µg/kg bodyweight, during 20 days of pregnancy) significantly lowers levels of brain-derived neurotrophic factor (BDNF) in fetal rat brain (18). BDNF is one of the four major types of neurotrophins. Neurotrophins are growth factors that play important roles in the regulation of the development and functioning of the nervous system but also in the regulation of the placenta during pregnancy (19). In Figure 1.3, the main roles of the four major neurotrophins (BDNF; nerve growth factor, NGF; neurotrophin-3, NT-3, neurotrophin-4, NT-4) during pregnancy are illustrated (19).

Alterations in neurotrophin levels are believed to affect neurotrophic processes, such as neuronal maturation and plasticity (19). Several studies on adverse effects of exposure to different environmental hazards have found relationships with BDNF. Saenen *et al.* (20) found that maternal exposure to particulate matter with a diameter ≤ 2.5 µm was inversely associated with *BDNF* gene expression in the human placenta. A positive association between cord blood BDNF levels and neurocognitive development in children 2 years of age was found in a Chinese cohort study on exposure to polycyclic aromatic hydrocarbons (PAHs) showing a statistically significantly inverse association between PAH-DNA adducts and BDNF levels (21). Another Chinese study reported on low-

level prenatal manganese exposure and its association with neurodevelopment in a mother-infant cohort (22). Manganese levels were inversely associated with BDNF levels in cord blood serum and under low levels of BDNF, manganese exposure was inversely associated with gross motor scores, adaptive scores and personal-social scores in children 1 year of age. Furthermore, BDNF levels showed to be positively associated with personal-social scores.

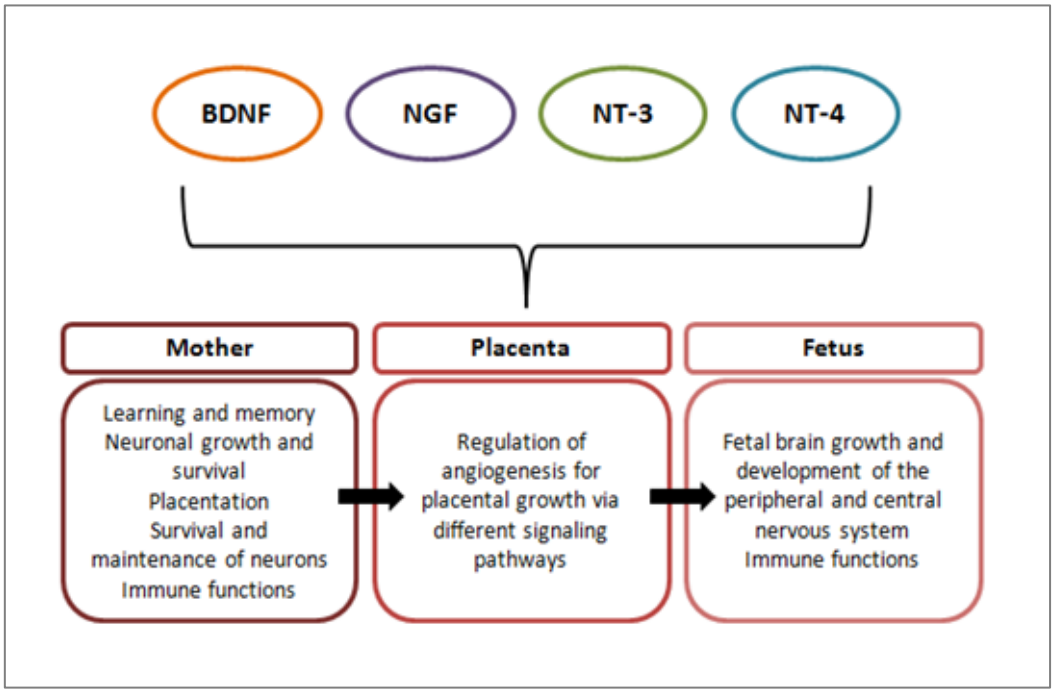

Figure 1.3. The role of the four major neurotrophins during pregnancy (adapted from Dhobale *et. al.*(19)). BNDF: brain-derived neurotrophic factor, NGF: nerve-growth factor; NT-3: neurotrophin-3; NT-4: neurotrophin-4.

The results of the abovementioned experimental and epidemiological studies give rise to the question whether maternal acrylamide exposure could affect human fetal BDNF levels. To the best of our knowledge, no epidemiological studies have been conducted on acrylamide exposure and adverse neurodevelopmental effects thus far.

1.6 Research objectives

Since nothing is known on the effects of acrylamide exposure on the human fetal nervous system and considering the exquisite susceptibility of the human developing brain *in utero*, studies on associations between acrylamide exposure *in utero* and neurological effects are urgently needed. The aim of this

study was to examine the association between prenatal acrylamide exposure and neurotrophin protein levels in cord blood, and the possible effect modification by genetic variations in acrylamide-metabolizing genes on this association. Therefore, we hypothesized that prenatal acrylamide exposure is inversely associated with BDNF levels in cord blood and that genetic variations in acrylamide-metabolizing genes modify this association. To address this hypothesis, two research objectives were formulated. First, the association between acrylamide hemoglobin (AA-Hb) adducts and BDNF levels in cord blood was investigated. Secondly, the effect of SNPs in genes involved in acrylamide metabolism on the association between AA-Hb adducts and BDNF levels was examined. The goal of the second objective was to get a better view on the causality of the investigated association. Genetic variants in acrylamide-metabolizing genes determine how well the human body metabolizes acrylamide, which in turn determines a person's susceptibility to acrylamide. Effect modifications of genetic variants on the association between acrylamide and BDNF, therefore, contribute to evidence for causality.

The European Food Safety Authority (EFSA) published its first full risk assessment of dietary acrylamide on June 4th, 2015 (23). According to EFSA's scientific opinion, possible harmful effects of acrylamide on pre- and postnatal development are not a concern for dietary exposure to acrylamide, based on extrapolations of animal data. As mentioned earlier, however, in the past few years epidemiological studies found inverse associations between dietary acrylamide exposure and markers of fetal growth (10-12). Nevertheless, the EFSA panel concluded that since there is no clear mechanism of action to explain the association between dietary acrylamide and adverse birth outcomes, the causality of these findings is unclear and that they could thus not use the human data in the risk assessment (23). However, the human data indicate that humans may be more sensitive to the developmental effects of acrylamide than animals.

Furthermore, neurological effects of dietary acrylamide exposure in fetal rats were also recently reported (18). The EFSA panel concluded that neurotoxicity is no human health concern. However, this conclusion was based on reference points from data of experimental animal studies, as data from epidemiological occupational studies were not sufficient for dose-response assessment according to the panel. There were no studies on dietary acrylamide exposure and neurotoxicity in humans.

2. MATERIALS AND METHODS

2.1 Study population

The current study is part of the ongoing population-based prospective birth cohort, ENVIR*ON*AGE (ENVIRonmental influence *ON* early AGEing), situated in the Flemish region of Belgium (15). Mother-newborn pairs have been recruited in the East-Limburg Hospital (Genk, Belgium) since February 2010 and recruitment is still ongoing. Exclusion criteria are the inability to fill out questionnaires in Dutch and planned caesarean sections. The general participation rate of eligible mothers is 61% and recruitment is spread approximately evenly over all seasons of the year.

The ENVIR*ON*AGE study was approved by the Ethics Committee of Hasselt University and East-Limburg Hospital. Participating mothers provided written informed consent upon arrival at the hospital for delivery and completed study questionnaires providing detailed information on place of residence, age, occupation, maternal education, parity, pre-gestational body mass index, smoking status, alcohol consumption, use of medication, and newborn's ethnicity, in the postnatal ward after delivery. Perinatal parameters that are collected after birth include birth date, newborn's sex, birth weight length and head circumference, gestational age, Apgar score and ultrasonographical data.

The main analysis in this study involved 70 singleton pregnancies selected from 633 mother-newborn pairs who were recruited between February 2010 and March 2014. This subset was obtained by including only participants of whom AA-Hb adduct levels were measured in umbilical cord whole blood samples. From these 70 mother-newborn pairs we excluded 1 preterm newborn, resulting in a final study population of 69.

2.2 Measurements of acrylamide hemoglobin adducts

Hemoglobin adduct levels of acrylamide in umbilical cord blood were determined by the Centers for Disease Control and Prevention (CDC) in the USA under supervision of Dr. H. Vesper. As part of their biomonitoring activities, the CDC developed a method specifically for high throughput screening, described by Ospina *et al*. (24). In short, this liquid chromatography tandem mass spectrometry (LC/MS/MS) method is based on the Edman degradation procedure using a fluorescent Edman reagent (pentafluorophenyl isothiocyanate, PFPITC) to detach adducts with N-terminal valines in hemoglobin, resulting in N-substituted valine derivatives that were isolated by protein precipitation and solid phase extraction. LC/MS/MS analysis was performed using acetic acid (0.1%) and electrospray ionization in a positive ion mode to separate and detect the Edman derivates.

2.3 Neurotrophin protein measurements

2.3.1 Protein measurements of brain-derived neurotrophic factor in umbilical cord plasma

Directly after delivery, umbilical cord blood was collected in BD Vacutainer™ Plastic Blood Collection Tubes coated with K_2EDTA (BD, Franklin Lakes, NJ, USA). To obtain plasma, samples were centrifuged at 3,200 rpm for 15 min and stored in Eppendorf® tubes at -80°C until further analysis.

Protein levels of BDNF were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) (Boster Human PicoKine™ ELISA kit; Boster Biological Technology, Pleasanton, CA, USA), according to the manufacturer's instructions. In short, 100 µl of samples and BDNF standards were added to an enzyme immunoassay plate, pre-coated with a mouse specific monoclonal antibody for BDNF. After 90 min of incubation at 37°C, 100 µl of biotinylated goat specific polyclonal antibody for BDNF was added to the plate, followed by an incubation period of 60 min. After washing 3 times with PBS, 100 µl of Avidin-Biotin-Peroxidase Complex, containing horseradish peroxidase (HRP), was added to all wells, followed by 30 min of incubation and 5 wash steps with PBS. Next, 90 µl of HRP substrate solution was added to obtain a blue color product during the last 30 min incubation period, which turned yellow after adding 100 µl of acidic stop solution. Finally, sample BDNF levels (pg/ml) were determined by comparing the absorbance values, measured at 450 nm, with the generated standard curve.

2.3.2 Development of a method to measure nerve growth factor, neurotrophin-3 and neurotrophin-4 protein levels in placental tissue

Protein levels of NGF, NT-3 and NT-4 were not detectable in our umbilical cord plasma samples using commercially available ELISA kits (Boster Human PicoKine™ ELISA kit; Boster Biological Technology, Pleasanton, CA, USA). Therefore, a method was developed to measure these neurotrophin levels in placental tissue using two types of placental tissue biopsies, as described below.

Within 10 min after delivery, placentas were collected and biopsies were taken according to a standardized protocol previously described by Adibi et al. (25). Briefly, placental tissue biopsies of approximately 1 cm^3 were taken at 4 equidistantly spread sites across the fetal side of the placenta, 4 cm from the umbilical cord and 1 – 1.5 cm below the chorioamniotic membrane. To minimize site variability, the largest umbilical cord artery served as a reference point to position the placenta facing upwards and the first biopsy was taken at the right side of the reference point. After washing with PBS, placental tissue samples were stored in Eppendorf® tubes containing RNALater (Qiagen, KJ Venlo, the

Netherlands), since biopsies were taken for multiple types of measurements. Samples were incubated at 4°C for 24 hours before storage at -20°C. Additionally, 3 pieces of placental tissue of approximately 5 x 3 cm were cut out of the placentae with a titanium knife, with the first piece cut out between the first and the second biopsy, the second piece between biopsy 3 and 4 and the third between biopsy 4 and 1. The decidua basalis and the chorionic plate were removed. Placental tissue pieces were drained from remaining blood using filter paper and transferred to metal-free falcon tubes, as these pieces are primarily used for metal concentration analyses. Tubes were stored at -20°C until further analysis.

To assess the level of neurotrophin proteins in placental tissue by obtained commercially available ELISA kits, both types of biopsies described above were used. After thawing, placental tissue biopsies were homogenized in chilled PBS (7.5 µl/mg tissue) containing cOmplete™ ULTRA Mini Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany) by using two stainless steel beads and the Retsch Mixer Mill MM 400 (Retsch, Haan, Germany). Tissue samples were disrupted 2 times for 2 min at 30 Hz and kept on ice for 5 min between and after disruption. Additionally, another set of biopsies were homogenized by sonicating 5 times in bursts of 10 s and kept on ice for 20 min, while using the same extraction buffer as mentioned above. After centrifugation of all samples at 16,000 x *g* for 20 min at 4°C, supernatant was aliquoted and stored at -20°C or used immediately for neurotrophin protein measurement by ELISA. Total protein concentrations were estimated with the Bio-Rad protein assay (Bio-Rad Laboratories, Temse, Belgium) according to the manufacturer's instructions.

2.4 Polymorphisms in acrylamide-metabolizing genes

2.4.1 Copy number variation in glutathione S-transferases

Genomic DNA was isolated from 200 µl of buffy coat using the QIAamp® DNA Mini Kit (Qiagen Inc., Venlo, The Netherlands), according to the manufacturer's instructions. In short, cells were lysed by using AL buffer and RNA and proteins were inactivated by RNAse A and proteinase K, respectively. To purify DNA, ethanol was added to the samples before applying them to the QIAamp Mini spin columns. Wash buffers and an elution buffer were used to ultimately collect the DNA from the samples. The concentration and purity of the isolated DNA were evaluated using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Mean DNA yield ± SD was 158.95 ± 92.56 ng/µl with an $A_{260/280}$ ratio of 1.87 ± 0.09 and an $A_{260/230}$ ratio of 2.17 ± 0.24. Extracted DNA samples were stored at -80°C until further analysis.

Copy numbers of *GSTM1* and *GSTT1* genes were determined with TaqMan Copy Number Assays (Applied Biosystems, Foster City, CA, USA). Briefly, assays were performed using an Applied

Biosystems™ 7900HT Fast Real-Time Polymerase Chain Reaction (PCR) System using 384 well plates with final reaction volumes of 10 µl containing 4 ng of genomic DNA, TaqMan® Genotyping Master Mix, Taqman Copy Number Assays (Hs02575461_cn or Hs00010004_cn) and Taqman Copy Number Reference Assay RNAse P (all from Applied Biosystems, Foster City, CA, USA). Samples were measured in triplicate and reactions were conducted using cycling conditions according to the manufacturer's instructions (95°C for 10 min hold, 40 cycles of 95°C for 15 s and 60°C for 60 s). Calibrator samples with a known copy number for *GSTM1* and *GSTT1* (Coriell Institute for Medical Research, Camden, NJ, USA) were included and at least 3 no template controls (NTCs) were used for each gene to assess contamination. After obtaining Ct values with SDS v2.3 software, copy numbers were determined using CopyCaller Software v2.1 (Applied Biosystems, Foster City, CA, USA). Minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines were followed.

2.4.2 Single nucleotide polymorphisms in placental tissue

Genomic DNA from placental tissue was also extracted by using the QIAamp DNA Mini Kit, as mentioned previously. Genotyping for SNPs in *CYP2E1*, *EPHX1* and *GSTP1* was performed at the Harvard Medical School-Partners Healthcare Center for Genetics and Genomics by using the Biotrove OpenArray SNP Genotyping Platform (26). In this study, only participants with a sample genotyping call rate ≥ 90% across all measured SNPs were included for analysis.

2.5 Statistical analysis

Statistical analyses were carried out using SAS 9.4 software (SAS Institute, Cary, NC, USA). Multiple linear regression analysis was performed to determine the association between acrylamide exposure and BDNF levels in umbilical cord blood. Covariables considered for entrance in the model ($p \leq 0.10$) included maternal age, maternal education (low/middle/high), pre-gestational body mass index (BMI), newborn's ethnicity (European/non-European), newborn's sex, birth weight, gestational age (weeks), parity (1/2/≥3), date of birth and smoking and alcohol use during pregnancy, and were selected with backward multiple linear regression procedures. Due to a correlation between BDNF levels and date of birth, corresponding with sample storage time, BDNF levels were standardized for this covariable. AA-Hb adducts were also associated with date of birth. To not remove the effect of acrylamide exposure, AA-Hb adduct levels were not corrected for date of birth. Of the other covariables only parity was associated with BDNF. In the final model, we adjusted for parity and gestational age, with the latter being forced into the model regardless of the p-value, since studies have shown that BDNF is correlated with gestational age (27). The Shapiro-Wilk test and QQ-plots of the residuals were used to

test the assumptions of linear models. Umbilical cord plasma acrylamide and BDNF levels were log_{10}-transformed to improve normality. Additionally, possible effect modification between acrylamide exposure and SNPs in acrylamide-metabolizing genes was studied by using interaction terms of acrylamide and the different genotypes (where callrate > 90%).

3. RESULTS

3.1 Characteristics of the study population

Characteristics of the 68 mother-newborn pairs that were included for the final statistical analysis are shown in Table 3.1. Mean maternal age (± SD) was 28.9 (± 4.0) years and pre-gestational BMI averaged 24.2 (± 5.1) kg/m^2. More than half of the participating mothers (54.4%, *n* = 37) obtained a degree in higher education, 27.9% (*n* = 19) reported to have smoked during pregnancy and 16.2% (*n* = 11) said to have consumed alcohol. The newborn population included 33 boys (48.5%), had a mean (± SD) gestational age of 39.3 (± 1.1) weeks and mean (± SD) birth weight of 3,400 (± 405) g. Most newborns were firstborns (47.1%, *n* = 32) or secondborns (44.1%, *n* = 30). The majority of newborns were of European origin (92.6%, *n* = 63). The median (IQR) of the measured umbilical cord AA-Hb adduct levels was 20.1 (17.3) pmol/g Hb. Cord plasma BDNF levels had a median (IQR) of 961 (873) pg/ml.

Table 3.1. Characteristics of mother-newborn pairs (*n* = 68)

Maternal characteristics	**Mean (± SD) or *n* (%)**	**Newborn characteristics**	**Mean (± SD) or *n* (%)**
Age, years	28.9 (4.0)	Sex	
Pre-gestational BMI, kg/m^2	24.2 (5.1)	Male	33 (48.5%)
Maternal education*		Female	35 (51.5%)
Low	10 (14.7%)	Ethnicity	
Middle	21 (30.9%)	European	63 (92.6%)
High	37 (54.4%)	Non-European	5 (7.4%)
Smoking during pregnancy		Gestational age, weeks	39.3 (1.1)
No	49 (72.1%)	Birth weight, grams	3,400 (405)
Alcohol consumption during pregnancy			
No	57 (83.8%)		**Median (IQR)**
Parity			
1	32 (47.1%)		
2	30 (44.1%)	Cord blood AA-Hb adducts, pmol/g Hb	20.1 (17.3)
≥ 3	6 (8.8%)	Cord plasma BDNF, pg/ml	961 (873)

* Maternal education was coded as low (no high school diploma), middle (high school diploma) and high (college or university degree).

Genotype frequencies of the SNPs and their corresponding *p*-value for Hardy-Weinberg equilibrium are presented in Table 3.2. None of the SNPs deviated statistically significantly from Hardy-Weinberg equilibrium. Furthermore, frequencies of *GSTM1* and *GSTT1* copy numbers are shown.

Table 3.2. Genotype frequencies and copy numbers of the study population

SNP ID	**Homozygous wild type, *n* (%)**	**Heterozygous, *n* (%)**	**Homozygous variant, *n* (%)**	**Hardy-Weinberg, *p* – value***
rs915906 (*CYP2E1*) *n* = 58	TT, 41 (70.7)	TC, 15 (25.9)	CC, 2 (3.4)	0.43
rs2480258 (*CYP2E1*) *n* = 59	CC, 38 (64.4)	CT, 19 (32.2)	TT, 2 (3.4)	0.99
rs11101888 (*CYP2E1*) *n* = 57	CC, 48 (84.2)	AC, 9 (15.8)	-	0.12
rs1051740 (*EPHX1*) *n* = 61	TT, 35 (57.4)	CT, 20 (32.8)	CC, 6 (9.8)	0.09
rs1695 (*GSTP1*) *n* = 61	AA, 23 (37.7)	AG, 31 (50.8)	GG, 7 (11.5)	0.87
rs1138272 (*GSTP1*) *n* = 59	CC, 48 (81.4)	CT, 10 (16.9)	TT, 1 (1.7)	0.48

Gene ID	**Copy number = 0, *n* (%)**	**Copy number ≥ 1, *n* (%)**
GSTM1, *n* = 68	42 (61.8)	26 (38.2)
GSTT1, *n* = 68	10 (14.7)	58 (85.3)

* Deviation from Hardy-Weinberg equilibrium was calculated using the full dataset of SNP measurements for the ENVIR*ON*AGE birth cohort (*n* = 741).

3.2 Prenatal acrylamide exposure in association with brain-derived neurotrophic protein levels in cord blood

Of the covariables shown in Table 3.1, BDNF levels were only associated with parity (-31.1%, 95% CI: -44.3 – -14.8%, $p < 0.001$). Gestational age was forced into the model even though this covariable was not statistically significantly associated with BDNF levels. In unadjusted analysis, umbilical cord blood plasma BDNF levels were inversely associated with *in utero* acrylamide exposure. BDNF levels decreased, borderline statistically significantly, by 16.9% (95% confidence interval (CI): -31.2 – 0.5%, $p = 0.06$) for every doubling in AA-Hb adduct levels. After adjusting for parity and gestational age the inverse association was still present, showing a decrease in BDNF levels of 15.4% (95% CI: -29.3 – 1.2%, $p = 0.07$) for every doubling in AA-Hb adduct levels (Figure 3.1).

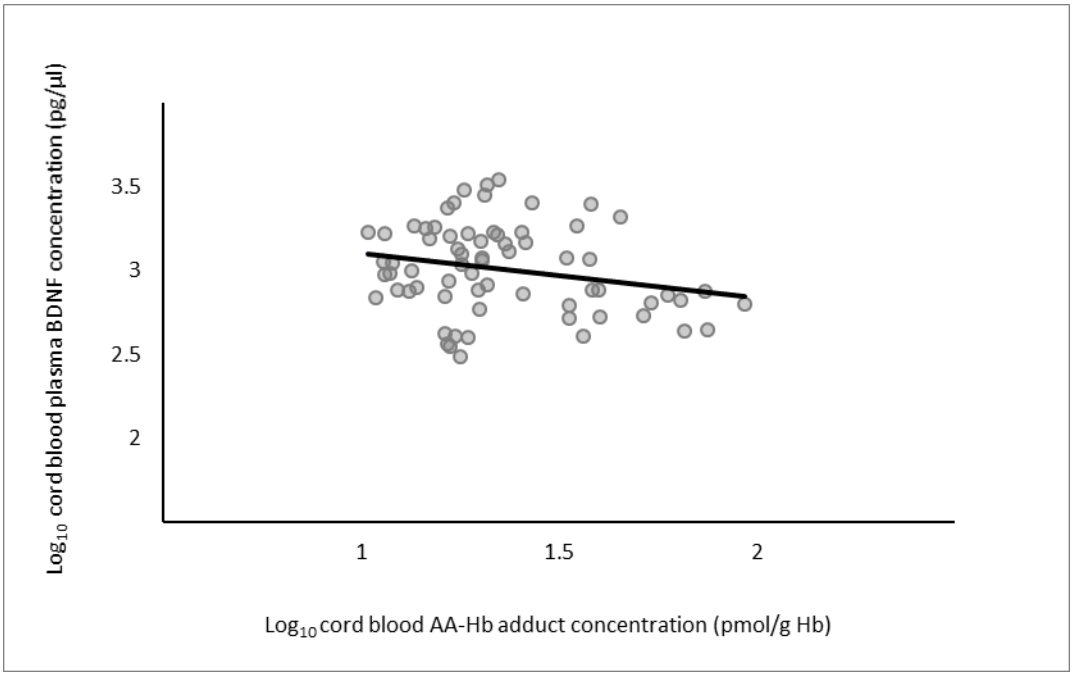

Figure 3.1. Scatterplot of $\log_{10}$ cord blood plasma BDNF levels in association with $\log_{10}$ cord blood acrylamide hemoglobin adducts (*n* = 68)

Subgroup analysis of non-smoking women showed no association between AA-Hb adducts and BDNF; levels of BDNF decreased by 5.2% (95% CI: -22.9 – 44.4%, *p* = 0.73) for every doubling in AA-Hb adduct levels. In boys (*n* = 33), a doubling in AA-Hb adducts non-significantly decreased BDNF levels with 11.5% (95% CI: -33.8 – 18.4%, *p* = 0.40), while BDNF levels in girls (*n* =35) decreased by 17.9% (95% CI: -38.3 – 9.1%, *p* = 0.17).

3.3 Main effects of single nucleotide polymorphisms and copy number variations on brain-derived neurotrophic factor protein levels

In Table 3.3, the independent associations between genotypes and BDNF levels are shown. None of the SNPs were statistically significantly associated with BDNF levels in cord blood plasma. Associations between copy number variants in *GSTM1* and *GSTT1* and BDNF levels were also not statistically significant. Newborns with a homozygous deletion of *GSTM1* had 3.6% (95% CI: -8.7 – 16.0%, *p* = 0.56) lower BDNF levels than newborns with at least 1 copy of the gene. No association was seen between *GSTT1* copy number and BDNF levels (-0.1%, 95% CI: -16.9 – 16.7%, *p* = 0.99).

Table 3.3. Main effects of SNPs and copy number variations on cord blood plasma BDNF levels

SNP ID*	**Estimated change (%) in cord blood plasma BDNF levels (95% CI)**
rs915906 (*CYP2E1*), *n* = 58	-0.4 (-14.2, 13.4)
rs2480258 (*CYP2E1*), *n* = 59	-5.2 (-18.1, 7.6)
rs11101888 (*CYP2E1*), *n* = 57	-14.0 (-31.9, 4.0)
rs1051740 (*EPHX1*), *n* = 61	-0.6 (-12.0, 13.2)
rs1695 (*GSTP1*), *n* = 61	7.1 (-5.5, 19.8)
rs1138272 (*GSTP1*), *n* = 59	3.0 (-13.6, 19.6)
Gene ID**	**Estimated change (%) in cord blood plasma BDNF levels (95% CI)**
GSTM1, *n* = 68	3.6 (-8.7, 16.0)
GSTT1, *n* = 68	-0.1 (-16.9, 16.6)

* homozygous wild type versus 1 or 2 variant alleles ** copy number = 0 versus copy number ≥ 1

Adjusted for parity and gestational age

3.4 Interactions between acrylamide exposure and single nucleotide polymorphisms or copy number variants

In Table 3.4, the interactions between SNPs and copy number variations and AA-Hb adducts on BDNF levels are shown. None of the SNPs in acrylamide-metabolizing genes statistically significantly modified the association between AA-Hb adducts and BDNF. Although statistically non-significant, several SNPs showed a stronger association between AA-Hb adducts and BDNF in one of the genotypes. Newborns with at least 1 variant allele of rs915906, rs2480258 or rs11101888 in *CYP2E1* showed a stronger inverse association between AA-Hb adducts and BDNF than newborns with homozygous wild type alleles, with a decrease in BDNF levels of respectively 32.0% (95% CI: -59.4 – 14.2%, *p* = 0.13), 26.8% (95% CI: -54.6 – 18.1%, *p* = 0.19) and 27.2% (95% CI: -59.1 – 29.5%, *p* = 020) for every doubling in AA-Hb adduct levels. A similar trend was seen for newborns with 1 or 2 variant alleles of rs1695 or rs1138272 in *GSTP1*. For every doubling in AA-Hb adducts, newborns with at least 1 variant allele of rs1695 had 22.0% (95% CI: -35.2 – -6.0%, *p* = 0.01) lower BDNF levels than newborns with homozygous wild type alleles and those with at least 1 variant allele for rs1138272 showed a decrease of 38.0% (95% CI: -56.1 – -12.6%, *p* = 0.01) in BDNF levels.

Table 3.4. Interactions between SNPs or copy number variations and AA-Hb adduct levels on cord blood plasma BDNF levels

SNP ID	Estimated change (%) in cord blood plasma BDNF levels (95% CI) for a doubling of AA-Hb adducts	*p* interaction
rs915906 (*CYP2E1*) = 0*, (*n* = 41) rs915906 (*CYP2E1*) = 1*, (*n* = 17)	-8.3 (-26.7, 14.6) -32.0 (-59.4, 14.2)	0.32
rs2480258 (*CYP2E1*) = 0, (*n* = 38) rs2480258 (*CYP2E1*) = 1, (*n* = 21)	-9.9 (-27.6, 12.2) -26.8 (-54.6, 18.1)	0.21
rs11101888 (*CYP2E1*) = 0, (*n* = 48) rs11101888 (*CYP2E1*) = 1, (*n* = 9)	-13.7 (-31.2, 8.2) -27.2 (-59.1, 29.5)	0.19
rs1051740 (*EPHX1*) = 0, (*n* = 35) rs1051740 (*EPHX1*) = 1, (*n* = 26)	-19.9 (-37.3, 2.3) -8.4 (-32.9, 24.9)	0.78
rs1695 (*GSTP1*) = 0, (*n* = 23) rs1695 (*GSTP1*) = 1, (*n* = 38)	-1.5 (-42.0, 67.3) -22.0 (-35.2, -6.0)	0.21
rs1138272 (*GSTP1*) = 0, (*n* = 48) rs1138272 (*GSTP1*) = 0, (*n* = 11)	-8.5 (-27.2, 14.9) -38.0 (-56.1, -12.6)	0.62
Gene ID	**Estimated change (%) in cord blood plasma BDNF levels (95% CI) for a doubling of AA-Hb adducts**	***p* interaction**
GSTM1 = 0**, (*n* = 42) *GSTM1* = 1**, (*n* = 26)	-20.6 (-35.7, -2.1) 3.0 (-28.5, 48.3)	0.12
GSTT1 = 0, (*n* = 10) *GSTT1* = 1, (*n* = 58)	-24.8 (-49.8, 12.5) -13.6 (-29.9, 6.4)	0.70

*0: homozygous wild type; 1: 1 or 2 variant alleles ** 0: copy number = 0; 1: copy number ≥ 1
Adjusted for parity and gestational age

Newborns with a homozygous deletion of *GSTM1* had statistically significantly decreased acrylamide-associated BDNF levels with 20.6% (95% CI: -35.7 – -2.1%, $p = 0.03$), whereas no clear increase (3.0%, 95% CI: -28.5 – 48.3%, $p = 0.87$) was seen for newborns with 1 or more copies of the gene but the interaction was not statistically significant (p interaction = 0.12). For *GSTT1* the same trend was seen, showing a decrease of 24.8% (95% CI: -49.8 – 12.5%, $p = 0.13$) in acrylamide-associated BDNF levels for newborns with a homozygous deletion.

3.5 Protein extraction from placental tissue biopsies

Since protein levels of NGF, NT-3 and NT-4 were not detectable in our cord blood plasma samples with the obtained commercially available ELISA kits, we optimized the extraction of proteins from placental biopsies for these measurements. Two different methods of tissue homogenization were tested:

disruption, using a tissue shredder, and sonication. In Figure 3.2, total protein concentrations (µg/ml) of 9 test samples using both methods are illustrated. Test samples were obtained according to the protocol used for obtaining placental tissue samples for analysis of metal concentrations, as described in Chapter 2.3.

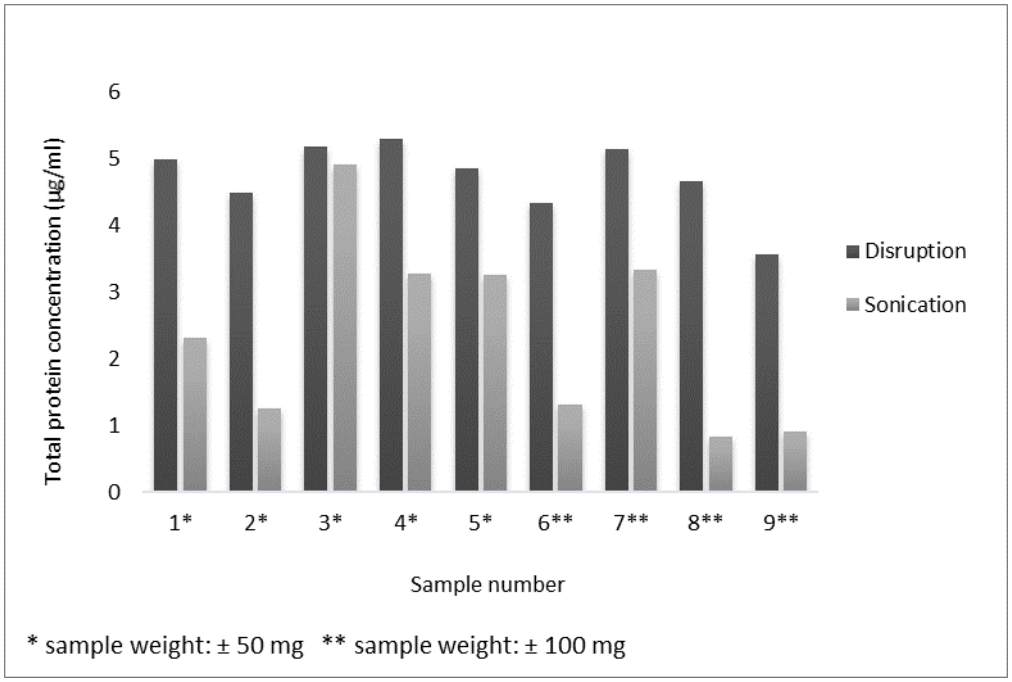

Figure 3.2. Histogram of total protein concentration of placental tissue samples homogenized by disruption and sonication. Protein concentrations were determined using the Bio-Rad protein assay

The mean (± SD) protein concentration of supernatant after disruption was 4.7 (± 0.5) µg/ml. Concentrations of supernatants of tissue biopsies of ± 50 mg averaged 5.0 (± 0.3) µg/ml and of biopsies of ± 100 mg averaged 4.4 (± 0.7) µg/ml. Protein concentrations of supernatants of sonicated samples had a mean (± SD) of 2.4 (± 1.4) µg/ml, with a mean of 3.0 (± 1.4) µg/ml for samples weighing ± 50 mg and 1.6 (± 1.2) µg/ml for samples of ± 100 mg. Since higher total protein concentrations were obtained by disrupting the samples with the tissue shredder, this method was used for further optimization.

Neurotrophins were detectable with the obtained ELISA kits in all 9 test samples homogenized by disruption. Mean (± SD) levels of NGF, NT-3 and NT-4 were 22.1 (± 8.1, *n* = 7), 13.1 (± 4.1, *n* = 5) and 8.8 (± 1.7, *n* = 4) pg/mg protein, respectively (Table 3.5).

Table 3.5. Total protein concentrations and neurotrophin levels (NGF, NT-3 and NT-4) of placental tissue samples originally obtained for analysis of metal concentrations.

Sample Nr	Total protein concentration (µg/ml)	NGF (pg/mg protein)	NT-3 (pg/mg protein)	NT-4 (pg/mg protein)
1	5.0	23.1	14.5	-
2	4.5	25.0	-	9.3
3	5.2	26.4	12.0	-
4	5.3	-	-	10.9
5	4.8	9.3	7.2	-
6	4.3	14.1	13.3	-
7	5.1	23.0	-	8.4
8	4.7	33.6	18.3	-
9	3.6	-	-	6.7

Neurotrophin, pg/mg protein	Mean (± SD)
NGF	22.1 (8.1)
NT-3	13.1 (4.1)
NT-4	8.8 (1.7)

Number of samples used for neurotrophin protein level measurements: NGF – *n* = 7; NT-3 – *n* = 5; NT-4 – *n* = 4.

Most placental biopsies that are taken for analyses within the ENVIR*ON*AGE birth cohort are stored in RNALater. These biopsies were also tested to determine if RNALater affects protein yield and quality. Total protein concentrations and neurotrophin levels of 15 test biopsies in RNALater are shown in Table 3.6. The average (± SD) total protein concentration was 3.9 (± 0.7) µg/ml. Mean neurotrophin levels were 39.3 (± 7.4), 16.0 (± 4.9) and 5.7 (± 2.9) pg/mg protein for NGF, NT-3 and NT-4, respectively.

Table 3.6. Total protein concentrations and neurotrophin levels (NGF, NT-3 and NT-4) of placental tissue samples stabilized in RNALater.

Sample Nr	Total protein concentration (µg/ml)	NGF (pg/mg protein)	NT-3 (pg/mg protein)	NT-4 (pg/mg protein)
1	3.8	39.2	21.0	10.8
2	2.7	49.8	18.0	7.8
3	2.5	43.6	14.7	2.1
4	4.2	41.2	20.2	9.4
5	3.5	29.3	9.1	1.6
6	4.7	44.2	21.4	10.4
7	4.0	52.9	26.6	5.5
8	3.0	42.1	14.3	5.5
9	4.4	31.1	10.1	3.1
10	3.8	41.5	15.4	6.2
11	3.9	35.6	16.7	6.6
12	4.8	33.6	12.8	4.2
13	4.7	25.5	9.0	2.7
14	4.6	37.8	14.2	4.8
15	4.5	41.5	16.4	4.8

Neurotrophin, pg/mg protein	Mean (± SD)
NGF	39.3 (7.4)
NT-3	16.0 (4.9)
NT-4	5.7 (2.9)

4. DISCUSSION

Exposure to environmental stressors during the fetal period of development can disrupt primary developmental processes that may lead to increased susceptibility to a variety of disorders later in life. This study (set in the ongoing ENVIR*ON*AGE birth cohort) examined the association between prenatal acrylamide exposure and BDNF levels in umbilical cord blood. The key finding of this investigation was that cord plasma BDNF levels borderline statistically significantly decreased by 15.4% (95% CI: -29.3 – 1.2%, p = 0.07) for every doubling in AA-Hb, after adjusting for parity and gestational age in multivariable-adjusted linear regression analysis.

BDNF is known to play a vital role in brain development and function, but also in learning, memory and behavior (28). Although they are still scarce, some recent studies reported that decreased cord blood BDNF levels were associated with cognitive impairment in infants, such as decreasing gross motor scores, adaptive scores and personal-social scores (21, 22). Thus, more research on prenatal acrylamide exposure and neonatal BDNF levels is very important. In addition, more research on the links between neonatal BDNF levels and long-term effects on cognition in childhood and adulthood is needed. In the ENVIRONAGE study, the link between neonatal BDNF levels and cognitive performance at 4 years of age will be examined in the near future.

To the best of our knowledge, our study was the first to investigate the association between prenatal acrylamide exposure and BDNF levels in humans. One recent experimental animal study by Erdemli *et al*. (18), examined the effects of acrylamide intake (5 mg/kg per day) during 20 days of pregnancy on the development of the fetal rat brain and BDNF levels in rat brain tissue. Additionally, they studied the possible protective role of vitamin E, a neuroprotective antioxidant, in counteracting the effects of acrylamide exposure. They found that compared to the control group, BDNF levels statistically significantly decreased in fetal brain tissue in the exposure group. In addition, acrylamide intake caused neurodegeneration and hemorrhagic damages, as well as statistically significantly decreased total antioxidant capacity and reduced glutathione levels. Administration of vitamin E together with acrylamide showed to statistically significantly increase BDNF and restore the abovementioned biochemical levels up to those of the control group. One of the mechanisms of action hypothesized to cause neurotoxicity due to acrylamide exposure is an imbalance between prooxidants and antioxidants in the body, also called oxidative stress. Experimental animal studies and *in vitro* experiments have shown that acrylamide exposure can induce oxidative stress by producing reactive oxygen species (ROS) and depleting glutathione, which occur when acrylamide is metabolized (29, 30). Oxidative stress can lead to numerous cell dysfunctions, including necrosis. In a pilot study by Naruszewicz *et al*. (31), examining effects of potato chips intake (4 weeks) in 14 individuals, ROS production by monocytes,

lymphocytes and granulocytes statistically significantly increased. A study in patients with bipolar disorder showed that blood serum thiobarbituric acid reactive substances, a byproduct of lipid peroxidation, were inversely associated with BDNF levels (32). Oxidative stress can, therefore, be a possible mechanism for the acrylamide-induced decrease in BDNF levels found in the current study, as well as in the rat study.

In the current study, genetic variations in genes involved in acrylamide metabolism were assessed to study their possible effect modification on the association between acrylamide exposure and BDNF levels in cord blood. We found a statistically significant decrease in acrylamide-associated BDNF levels in newborns with a homozygous deletion of *GSTM1* but no association in newborns with 1 or more copies of the gene. GSTs detoxify numerous environmental pollutants, including acrylamide and its main metabolite glycidamide, and therefore have an important role in the protection against oxidative stress (33). Duale *et al*. (8) found that individuals with a homozygous deletion of *GSTM1*, as well as a deletion of *GSTT1*, had increased levels of AA- and GA-Hb adducts. Additionally, these individuals showed a statistically significantly higher ratio of GA-Hb/AA-Hb adducts than individuals with 1 or more GST alleles. GSTM1 and GSTT1 are both important in the main route of acrylamide excretion by conjugation of acrylamide and glycidamide to glutathione. Therefore, individuals without enzymatic activity because of null genotypes, and thus decreased excretion of acrylamide and glycidamide, are more susceptible to adverse health outcomes. In our study, newborns with a homozygous deletion of *GSTT1* also had decreased acrylamide-associated BDNF levels and the decrease was stronger than in newborns with 1 or more copies of the gene, although not statistically significant. Another polymorphism known to decrease enzymatic activity is the 105Val allele polymorphism (rs1695) in *GSTP1*. We found that newborns with 1 or 2 variant alleles of rs1695 had statistically significantly decreased BDNF levels but newborns with homozygous wild type alleles did not show an association. The same pattern was seen for rs1138272 in *GSTP1*. These findings again suggest that oxidative stress lies at the basis of acrylamide-associated decreased BDNF levels.

The enzyme CYP2E1 is known to metabolically activate a variety of toxic compounds, including acrylamide. Ghanayem *et al*. (34), demonstrated in *CYP2E1*-null mice, that CYP2E1 is the key enzyme in the conversion of acrylamide to its epoxide metabolite glycidamide. Levels of blood plasma acrylamide were 137-fold higher in *CYP2E1*-null mice after administration of acrylamide than in wild type mice and glycidamide levels were only detectable in wild type mice. Additionally, AA-Hb adduct levels were significantly higher in *CYP2E1* knockouts than in wild type mice and no glycidamide or glycidamide derivates were detectable in urine of *CYP2E1*-null mice. In rodents, deficient CYP2E1 enzyme function greatly diminishes conversion of acrylamide to the genotoxic metabolite glycidamide. This conclusion was confirmed for humans in an *in vitro* study (35) using human CYP-expressing cell

cultures, as well as in an *in vivo* study (36), in which healthy volunteers were phenotyped for CYP2E1 after a single ingestion of potato chips containing 1 mg acrylamide. In our study, we found stronger (albeit non-statistically significant) reduced acrylamide-associated BDNF levels in newborns with 1 or 2 variant alleles of rs915906 and rs2480258in *CYP2E1* than in newborns with homozygous wild type alleles. Although a lot of research has been performed on other SNPs in *CYP2E1* in relation to the risk of different diseases, not much is known about the SNPs in the current study. A recent study by Pellé et al., found that the rs2480258 allele was associated with a reduced CYP2E1 phenotype on the mRNA and protein level, as well as on the level of enzyme activity (37). Over the years, the body of evidence showing that acrylamide itself is responsible for acrylamide-induced neurotoxic effects has grown (3, 38, 39). The stronger association between AA-Hb adducts and BDNF levels for newborns with variant alleles of rs2480258 seen in the current study would suggest that acrylamide is the compound causing decreased BDNF levels. On the other hand, in recent studies by Hogervorst *et al*. (40), a stronger association between acrylamide and endometrial and ovarian cancer risk was found in homozygous wild types for rs2480258 and rs915906. These findings, together with the findings of Pellé *et al*., support the hypothesis that glycidamide is the causative compound in acrylamide-induced carcinogenesis. Even though the effect modifications by genetic variations that we investigated in the current study were statistically non-significant and might be a result of chance, the abovementioned SNPs and copy number variants are worthwhile to examine in future studies, along with SNPs in vital oxidative stress-related genes.

In our study, NGF, NT-3 and NT-4 were not detectable in umbilical cord plasma with a commercially available ELISA kit. Different methods were tested to optimize detection, such as concentrating plasma proteins with help of filter devices and using cord serum samples instead of cord plasma, as the EDTA in our cord plasma samples can inhibit HRP (41). Unfortunately, these methods showed no success. Next to their function in the regulation of neuronal survival, proliferation and differentiation in the nervous system, neurotrophins have shown to be vital in the growth and development of the placenta, as well as of the fetus, by having roles in the regulation of angiogenesis and energy homeostasis (28). Research shows that neurotrophins can be transported to the fetus from the maternal circulation but are also produced in the human placenta (28, 42). Several studies have detected neurotrophin proteins in human placenta (43, 44). We therefore decided to develop a method to measure neurotrophins in placental tissue. Sahay *et al*. (43) found average NGF concentrations of 15.75 (± 1.7) pg/mg protein in the central fetal side of the placenta and 13.5 (± 1.4) pg/mg total protein in the peripheral side. Test results of our placental biopsies showed an average (± SD) NGF concentration of 22.1 (± 8.1) pg/mg protein (biopsies taken for metal concentration measurement) and 39.3 (± 7.4) pg/mg protein (biopsies stored in RNAlater). Differences in averages between our study and that of Sahay may be

explained by the difference in the number of placentae examined and possible outliers, since Sahay *et al*., had a larger study population (*n* = 50). To our knowledge, no other studies have investigated NT-3 and NT-4 protein levels in placental tissue by ELISA. Unexpectedly, we found higher concentrations of NGF, NT-3 and NT-4 in biopsies that were stored in RNAlater, which stabilizes RNA and proteins but which may also denature proteins. However, Reiser *et al*. found that preservation of tissues in RNAlater is suitable for ELISA (45). Even though the methods of placental tissue sampling were standardized by using fixed locations, biopsies of multiple locations were used for our pilot study. It has been suggested that differences in the architecture and blood flow of the placenta lead to regional differences of oxidative stress which in turn may lead to regional differences in a variety of growth factors (28). Overall, we were able to detect NGF, NT-3 and NT-4 in placental tissue biopsies with a commercially available ELISA kit. To correct for regional differences in placental tissue, the use of pooled biopsies is advised for future experiments.

Cord plasma BDNF levels that we measured fall into the range of levels found in similar studies. We found BDNF levels with a median (IQR) of 961 (873) pg/ml. Median levels of two of the most recent studies were 1321 (985) pg/ml (46) and 645 (487) pg/ml (27). Furthermore, AA-Hb adducts were measured under supervision of Dr. H. Vesper by using a strictly protocolled high-throughput LS/MS/MS method that was specifically developed by the CDC for screening big populations. However, Dr. Vesper had no experience with this method for cord blood samples and thus has to optimize the methodology for these samples for future measurements. In our pilot study, only AA-Hb adduct level measurements were of sufficient quality. AA-Hb adduct levels, together with GA-Hb adduct levels, are considered to be a biomarker for internal acrylamide dose over the lifespan of the erythrocyte. In future studies, we recommend to also obtain GA-Hb adduct levels to be able to comprehensively study the association between acrylamide exposure and BDNF and the role of genetic variations in enzymes involved in acrylamide-metabolism. AA-Hb adduct levels reflect acrylamide exposure over approximately the last 4 months. It has been reported that BDNF is especially important in the development of the placenta and growth of the fetus from mid gestation to late gestation in mice (28), which makes AA-Hb adduct levels a fitting biomarker in this study. Our study also has some limitations. Often, to obtain meaningful results that are of statistical significance in epidemiological research, large study populations are required. Our study population was rather small due to the suboptimal quality of a portion of the AA-Hb adduct measurements, which resulted in a loss of one third of the original study population. Nevertheless, the association we found between acrylamide exposure and BDNF levels in cord blood was borderline statistically significant. Due to the relatively small study population, statistical power was possibly too low to detect statistically significant differences in the association between AA-Hb adducts and BDNF levels in the different genotype subgroups that were studied. In future studies,

therefore, the use of larger study populations is recommended. Even though smoking is an important source of acrylamide exposure, we did not adjust for smoking in our multivariate model, since smoking was not associated to BDNF in our study population. When we restricted our analysis to newborns of non-smoking women, we did not see an association between acrylamide exposure and BDNF. This is probably due to the fact that a substantial amount of the variation in AA-Hb adduct levels is lost when newborns of non-smokers are selected because smoking is such an important source of acrylamide exposure. Because smoking was not associated with BDNF, we are confident that the association between acrylamide and BDNF is not due to confounding by smoking.

We found a statistically significant positive association between AA-Hb adduct levels and time since birth and an inverse association between BDNF levels and time since birth. This means that BDNF levels decrease with the duration that the samples have been stored and that AA-Hb levels increase with longer storage time. For AA-Hb, we decided not to adjust for date of birth in the multivariable-adjusted models, since over the years the food industry has put effort in decreasing acrylamide concentrations in common foods and thus the intake of acrylamide may have declined. This may explain the decrease in AA-Hb adduct levels that we observed between 2010 and 2014 (6). If we did correct for date of birth, we would also correct for the effect of the exposure. Additionally, samples stored at -70°C are stable for at least 5 years and are not expected to decrease in AA-Hb adduct levels with time (47). BDNF levels were, however, normalized for date of birth before running the multivariable-adjusted models, since the possibility exists that proteins, such as BDNF, decrease with storage time and freeze-thaw cycles (48). Lastly, unmeasured and residual confounding by variables associated with both AA-Hb adduct levels and BDNF levels in cord blood is always possible in observational settings such as this study.

5. CONCLUSION

The goal of this study was to examine whether prenatal acrylamide exposure was associated with neonatal BDNF levels and whether genetic variants in acrylamide-metabolizing genes modify this association. We found a borderline statistically significant inverse association between AA-Hb adduct levels and BDNF levels in cord blood.

None of the interactions between acrylamide and genetic variants were statistically significant in our study. However, we did find a statistically significant decrease in acrylamide-associated BDNF levels in newborns with a homozygous deletion of GSTM1, while there was no association among newborns with 1 or more copies.

Furthermore, we observed stronger inverse associations between AA-Hb and BDNF in newborns with variant alleles of rs915906 and rs2480258 in *CYP2E1*, and rs1695 in *GSTP1*. To the best of our knowledge, we are the first to report on the association between acrylamide exposure and BDNF in humans.

Our results suggest that *in utero* exposure to acrylamide is inversely associated with neonatal BDNF levels. Considering our findings on the interaction of acrylamide with genetic variants in genes that are important in the defense against ROS, we propose that oxidative stress may be a vital mechanism causing decreased acrylamide-induced BDNF levels.

Although scarce, there is literature that suggests that cord blood BDNF levels may be a risk factor for impaired cognitive development in children. Therefore, we recommend more and larger studies on the association between prenatal acrylamide exposure and neonatal BDNF levels with attention to interactions with acrylamide-metabolizing and vital oxidative stress-related genes.

Since acrylamide is present in a variety of common foods and considering the susceptibility of the fetal developing brain, the results of this study, if confirmed, could provide important information for improved risk assessment, future evidence-based policy making and health education for pregnant women.

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
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