Detection of a Hemoglobin Adduct of the Food Contaminant Furfuryl Alcohol in Humans: Levels of N-((Furan-2-yl)methyl)-valine in Two Epidemiological Studies

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Scope: Furfuryl alcohol is a heat-induced food contaminant, classified as possibly carcinogenic to humans. The proximal carcinogen 2-sulfoxyethylfurane leads to adduct formation in DNA and proteins (e.g., N-((furan-2-yl)methyl)-Val (FFA-Val) in hemoglobin).

Methods and results: This study analyzed human erythrocyte samples from two studies for the presence of FFA-Val: the Risks and Benefits of a Vegan Diet study (RBVD; 72 adults) and the ENVIRONMENTAL INFLUENCE ON early AGEING birth cohort study (ENVIRONAGE; 100 mother-newborn pairs). In the RBVD study, FFA-Val levels are lower in vegans compared to omnivores (median 13.0 vs 15.8 pmol g⁻¹ hemoglobin, *p* = 0.008), and lower in non-smokers compared to smokers (median 14.1 vs 17.0 pmol g⁻¹ hemoglobin, *p* = 0.003). In the birth cohort, FFA-Val levels are distinctly higher in maternal compared to newborn samples (median 15.2 vs 2.2 pmol g⁻¹ hemoglobin, *p* < 0.001).

Conclusions: FFA-Val, hitherto detected only in blood samples of mice, is quantifiable in all human samples, indicating a general exposure to furfuryl alcohol. The low adduct levels in blood samples from newborn children suggested that the placenta is a barrier to furfuryl alcohol. Dietary habits and tobacco smoking are two main influencing factors on the formation of FFA-Val, which may be of use as a biomarker of exposure to furfuryl alcohol.

1. Introduction

Furfuryl alcohol is a heat-induced food contaminant, which originates, for example, from acid-catalyzed and thermal dehydration of pentoses[1] and from glucose in the Maillard reaction.[2] It is considered as possibly carcinogenic to humans (International Agency for Research on Cancer [IARC] group 2B).[3] The classification is based mainly on the observation of carcinogenic effects in a 2-year inhalation study of the National Toxicology Program (NTP), i.e., increased incidences of renal tubule neoplasms in male B6C3F1 mice and nasal neoplasms in male F344/N rats.[4] The collected data from mutagenicity testing in vitro and in vivo were contradictory. The EFSA stated that most studies were negative, although some positive results were reported.[5] For example, chromosomal aberrations in Chinese hamster ovary (CHO) cells increased in a concentration-dependent manner, the effect being enhanced in the presence of rat liver S9 mix.[6] In contrast, furfuryl alcohol was unobtrusive in standard assays with Salmonella typhimurium strains TA98, TA100, and TA102.[7,8] The chromosomal aberrations test in CHO cells was negative in the absence of S9 and equivocal in the presence of S9 in the NTP study, and no sister chromatid exchanges or micronuclei were detected in bone marrow cells of male B6C3F1 mice after intraperitoneal injection.[4]

The mechanism underlying its carcinogenic effect may originate from sulfo conjugation of the alcohol moiety resulting in the formation of a reactive sulfate ester, which is prone to react with cellular nucleophiles (Figure 1).[9,10] Confirming this hypothesis, furfuryl alcohol was mutagenic in S. typhimurium TA100 engineered for expression of human sulfotransferase (SULT) 1A1, but not in the parental strain. The sulfo conjugation of furfuryl alcohol yielded the reactive 2-sulfoxyethylfurane (2-SMF), which in turn led to the formation of the DNA adducts N⁶-((furan-2-yl)methyl)-2′-deoxyadenosine (N⁶-MFdA) and N⁶-((furan-2-yl)methyl)-2′-deoxyguanosine (N⁶-MFdG). These were
also detected in DNA samples isolated from liver, lung, and kidney of FVB/N mice after daily exposure to furfuryl alcohol in a sub-acute study,[10] and in the tissues of mice expressing human SULT1A1/1A2 instead of mouse Sult1a1.[31] In addition, N\(^{-}\)-((furan-2-yl)methyl)-2'-deoxyguanosine (N\(^{2}\)-MF-dG in DNA) or N\(^{-}\)-((furan-2-yl)methyl)-Val (FFA-Val in hemoglobin).

Furfuryl alcohol is found in many foods. A high average content of 251 mg kg\(^{-1}\) was detected in coffee beans (\(n = 30\)),[30] and the concentrations in the samples of brewed coffee of different brands (\(n = 11\)) were between 32.9 and 88.7 mg L\(^{-1}\).[2] Relatively high amounts of furfuryl alcohol were also found in barrel-aged red wine (\(n = 8\), mean 6.3 mg L\(^{-1}\)) and white wine (\(n = 8\), mean 3.6 mg L\(^{-1}\)).[14] Balsamic vinegar (\(n = 19\); range: 4.9 – 65.7 mg kg\(^{-1}\)),[15] rice cakes (\(n = 2\); 2.0 and 2.3 mg kg\(^{-1}\)),[16] and white onions (18.3 – 157 mg kg\(^{-1}\), depending on the harvest time).[17] Low amounts were detected in jarred baby food (\(n = 24\); range: 9.9 – 21.3 μg kg\(^{-1}\)[18]) or in milk and different milk products, e.g., cheese (\(n = 41\); range: 9.9 – 21.3 μg kg\(^{-1}\)), sour cream (\(n = 11\); range: 9.9 – 21.3 μg kg\(^{-1}\)), and yogurt (\(n = 9\); range: 9.9 – 21.3 μg kg\(^{-1}\)[19]). It was also detected in a wide range of other food classes, albeit at lower amounts, and usually the sample sizes were very small.[31] Due to the limited data on furfuryl alcohol contents in food it is not possible to estimate reliably the human dietary intake. Additional sources may contribute with unknown quantities to the overall exposure. For example, high levels of furfuryl alcohol were found in 340 particulate matter samples out of 389 residences in Sweden (range: 0.4 – 500 μg g\(^{-1}\) dust).[20] in cigarette smoke (\(n = 4\); range 18 – 65 μg per cigarette in the mainstream smoke),[21] and in water pipe smoke (\(n = 5\); range: 55.7 – 552 μg per session).[22]

Recently, we developed a new technique that allows monitoring of the internal furfuryl alcohol exposure using erythrocyte samples. The hemoglobin adduct N\(^{-}\)-((furan-2-yl)methyl)-valine (FFA-Val; Figure 1) is quantified after cleavage with fluorescein-5-isothiocyanate (FITC) in a modified Edman degradation (FIRE procedure developed by Rydberg et al.[21]) using isotope-dilution ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The technique allowed studying the role of mouse and human SULT[24] and the influence of ethanol intake[25] on the bioactivation of furfuryl alcohol in different mouse models. The primary goal of the current work was to test whether FFA-Val is also detectable in human blood samples. We analyzed FFA-Val in hemoglobin of 100 mother per newborn pairs enrolled in the ENVIROmental influence ON early AGEing (ENVIRONAGE) birth cohort study,[26] and of 72 adults of the cross-sectional Risks and Benefits of a Vegan Diet (RBVD) study.[27]

2. Results

2.1. Quantification of FFA-Val in Human Blood Samples by UHPLC-MS/MS

An isotope-dilution technique was used to determine FFA-Val levels in hemoglobin following a modified Edman degradation of the N-terminal Val (Figure S1, Supporting Information). This method using FITC as a cleavage reagent was developed by Rydberg et al. (FIRE procedure).[23] The resulting conjugate FFA-Val-FTH yielded an intense peak at the fragmentation \(m/z\) 569.1 \(→\) 390.1 (retention time \(≈\) 14.9 min), which was used as quantifier signal. The specific cleavage of the 2-methylfuran cation \((m/z 569.1 \rightarrow 81.0)\) served as qualifier signal. The sensitivity of detection was relatively high, because the hydrophobicity of the adduct conjugate FFA-Val-FTH facilitated the chromatographic separation from the more polar byproducts of the FITC-mediated Edman cleavage, which interfere with the detection of more hydrophilic adducts like, e.g., N-(2,3-dihydroxypropyl)-Val formed from glycidol.[28] However, the limits of detection and quantification (LOD and LOQ) for this technique were not determined easily due to the presence of FFA-Val in all human blood samples. Based on the signal of the fragmentation \(m/z\) 576.1 \(→\) 81.0 of the isotope-labeled standard, the LOD and LOQ were estimated to be 0.8 fmol FFA-d7-Val-FTH on column (\(S/N = 4.0\)), corresponding to 0.2 pmol FFA-d7-Val-FTH g\(^{-1}\) hemoglobin, and 4.0 fmol FFA-d7-Val-FTH on column (\(S/N = 10.8\)), corresponding to 0.9 pmol FFA-d7-Val-FTH g\(^{-1}\) hemoglobin, respectively.[25] In the current study, the \(S/N\) of the quantifier peak \((m/z = 569.1 \rightarrow 390.1)\) in individual samples was used to judge the suitability for the calculation of FFA-Val-FTH, which was possible for all of the 272 samples from both studies. The \(S/N\) values of the four cord blood samples with the lowest adduct levels (range: 0.63 – 1.23 pmol FFA-Val per g hemoglobin) were in the range between 17 and 62. Example analyses of two blood samples with different FFA-Val content are shown in Figure 2.

The intraday precision was calculated by analyzing three concentrations of FFA-d7-Val-FTH (0.2, 1, and 5 nM) dissolved in post-processed Edman matrix (\(n = 5\)). The interday precision values were determined with the same sample sets prepared in different weeks (\(n = 5\)). The CV% of the intraday precision were 5.9 (0.2 nM), 8.3 (1 nM), and 10.2 (5 nM). The CV% of the interday precision were 8.2 (0.2 nM), 5.3 (1 nM), and 7.1 (5 nM).

2.2. FFA-Val in Blood Samples of Mother–Infant Pairs

FFA-Val was determined in the blood samples of 100 pairs of mothers and newborn infants from the ENVIRONAGE birth cohort (Figure 3). All FFA-Val levels were above the LOQ. The
Figure 2. UHPLC-MS/MS chromatograms of FFA-Val-FTH after an Edman degradation of a maternal blood sample a, b) and of the cord blood sample of the respective newborn child c, d). The signal (retention time ∼ 14.9 min) reflecting the amount of FFA-Val-FTH in the blood sample of the mother (m/z 569.1 → 390.1, panel a) is about six times more intense compared to that observed in the blood sample of the newborn (panel c). The panels b and d on the right hand side show the signals of the internal isotope-labeled standard FFA-d7-Val-FTH (m/z 576.1 → 390.1). The ratio of peak areas for the transition 569.1 → 390.1 (FFA-Val-FTH) and for the transition 576.1 → 390.1 (FFA-d7-Val-FTH) was used to calculate the FFA-Val content of the hemoglobin samples.

Figure 3. Levels of FFA-Val in blood samples of mothers, in the subsets of reported non-smoking and smoking mothers and in their respective newborns' blood samples from the ENVIRONAGE birth cohort.[26] Lines and boxes represent the median values and the lower and upper quartiles, respectively, and the error bars represent the 10th and 90th percentiles. The FFA-Val levels detected in blood samples of mothers and of the respective newborns were significantly different (p < 0.001, Mann–Whitney rank-sum test).

characteristics of the mothers and of the newborns are summarized in Table S1 of the Supporting Information. The difference between the levels of FFA-Val in maternal blood among all mothers (median 15.2, IQR 12.5–18.2 pmol g⁻¹ hemoglobin) and in the cord blood samples (median 2.2, IQR 1.7–2.8 pmol g⁻¹ hemoglobin) was statistically significant (p < 0.001). There was a weak association (r = 0.17, p = 0.088) between FFA-Val levels in cord and maternal blood samples of all mother–newborn pairs (Figure S2, Supporting Information). The median of all ratios of adduct levels (cord to maternal blood) was 0.15 (IQR 0.11–0.19) pmol g⁻¹ hemoglobin.

In blood samples from mothers who self-reported to be a non-smoker (n = 85), the median adduct level was 15.3 (IQR 12.5–18.3) pmol g⁻¹ hemoglobin. In cord blood samples of neonates from non-smoking mothers, the median was 2.3 (IQR 1.8–2.9) pmol g⁻¹ hemoglobin (Figure 3). Blood samples of mothers who smoked during pregnancy and cord blood samples of their newborns (n = 6) contained median FFA-Val levels of 18.1 (IQR 16.7–20.0) and 1.9 (IQR 1.5–8.2) pmol g⁻¹ hemoglobin, respectively. There was a clear difference between the adduct levels in smoking and non-smoking mothers (p = 0.052, Figure 3), but no difference in case of the cord blood samples of the respective newborns (p = 0.57). There were no significant associations between FFA-Val and any of the other variables in Table S1, Supporting Information.

2.3. FFA-Val in Blood Samples of Vegans and Omnivores

FFA-Val was well quantifiable in all erythrocyte samples from the RBVD study[27] on the health effects of a vegan diet (Figure 4). The participants’ characteristics are summarized in Table S2 of the Supporting Information. The median level of FFA-Val of all participants was 14.9 (IQR 12.4–17.0) pmol g⁻¹ hemoglobin. There was a small but significant difference (p = 0.008) between the adduct levels observed in vegans (median 13.0, IQR 11.7–16.6 pmol g⁻¹ hemoglobin) and omnivores (median 15.8, IQR 13.7–17.6 pmol g⁻¹ hemoglobin). Adduct levels did not differ (p = 0.76) between women (median 14.9, IQR
12.2–17.2 pmol g\(^{-1}\) hemoglobin) and men (median 14.9, IQR 12.4–16.9 pmol g\(^{-1}\) hemoglobin). FFA-Val levels were significantly lower in non-smokers (\(n = 59\), median 14.1, IQR 12.0–16.6 pmol g\(^{-1}\) hemoglobin) than in smokers (\(n = 13\), median 17.0, IQR 15.4–19.4 pmol g\(^{-1}\) hemoglobin) (\(p = 0.003\)), one of which reported to smoke exclusively cigars. Apart from the smoking habits and diet, FFA-Val levels were not associated with any other of the characteristics summarized in Table S2 (Supporting Information), such as age or BMI. The correlation analysis between FFA-Val levels in hemoglobin samples of vegans or omnivores and those of non-smokers and smokers was significantly different (\(\star \star \star \ p < 0.005\), Mann–Whitney rank-sum test).

### 3. Discussion

Some techniques based on the Edman degradation of modified Val residues from the N-termini in hemoglobin have already been developed for the analysis of the internal exposure to heat-induced contaminants in food, e.g., for acrylamide, glycidamide, and glycidol, and their applicability in human biomonitoring was proven by controlled exposure studies.[28,29] Recently, we established an isotope-dilution UHPLC-MS/MS method for the quantification of FFA-Val, a possible biomarker of exposure to furfuryl alcohol. Studies in mice showed that administration of furfuryl alcohol leads to the formation of FFA-Val, however, a low background level was also present in hemoglobin samples of untreated control animals.[24,25]

In the present work, we quantified FFA-Val also in erythrocytes of all human adults from two different cohorts and significantly lower levels in the umbilical cord blood of newborns. The current data indicated that dietary habits and tobacco smoking are two main influencing factors on the FFA-Val formation. The effect of smoking on FFA-Val formation was evident in both studies. However, it was not possible to determine a quantitative relationship between the number of cigarettes smoked per day and the FFA-Val levels based on the available data. This was mainly due to the circumstance that the daily cigarette consumption was not recorded exactly in the RBVD study (smoking habits were roughly defined in three classes, low (<10 cigarettes per day; \(n = 10\)), average (10–19 cigarettes per day; \(n = 1\)), and high (20 or more cigarettes per day; \(n = 1\)), and that smoking had a relatively small effect on the comparatively wide range of FFA-Val levels. The difference between the FFA-Val levels in smokers and non-smokers was significant (Figures 3 and 4). Considering “cigarette smoking” as a single parameter (the mean reported exposure in the birth cohort study was 9.2 cigarettes per day, \(n = 6\)), the comparison of median FFA-Val levels in smoking versus non-smoking mothers indicated that “cigarette smoking” led to an estimated FFA-Val increase of 18%. In the RBVD study, the median FFA-Val levels in blood samples from cigarette smokers (median = 16.9 pmol g\(^{-1}\) hemoglobin, \(n = 12\)) was 21% higher than the median level of all non-smokers regardless of their diet. The alteration of adduct levels caused by other compounds in cigarette smoke, which are also common food contaminants, is much larger. The levels of the hemoglobin adducts of acrylamide and glycidamide were about 3-fold and about 2.3-fold higher, respectively, in smokers (\(n = 255\)) than those observed in non-smokers (\(n = 255\)) from the European Union.[10] Similarly, Aasa et al.[31] reported that there was a considerable difference between the mean levels of hemoglobin adducts resulting from glycidol exposure between non-smoking (10.3 pmol g\(^{-1}\) hemoglobin, \(n = 6\)) and smoking adults (23.4 pmol g\(^{-1}\) hemoglobin, \(n = 6\)), however, the number of cigarettes per day was not reported. Taken together, despite its presence in the mainstream smoke of cigarettes,[21] smoking seems to contribute less to the overall exposure of furfuryl alcohol compared to respective contributions from that of the other mentioned tobacco smoke components.

The difference of FFA-Val levels between vegans and omnivores suggested that some characteristic features of the diets alter the furfuryl alcohol exposure. The correlation between FFA-Val levels and coffee consumption supported previous observations suggesting that coffee is a major source of furfuryl alcohol.[22,33] Except for coffee, there were no relevant correlations between FFA-Val levels in blood samples and the intake of single food groups in the RBVD study. This may be due to various reasons. First, furfuryl alcohol exposure results from a broad spectrum of sources and, second, their contribution to the overall exposure may not be easily entangled with a small number of study participants. Third, the time intervals of the exposure biomarker and the FFQ did not match: FFA-Val reflects the approximate exposure in the birth cohort study was 9.2 cigarettes per day, \(n = 6\), the comparison of median FFA-Val levels in smoking versus non-smoking mothers indicated that “cigarette smoking” led to an estimated FFA-Val increase of 18%. In the RBVD study, the median FFA-Val levels in blood samples from cigarette smokers (median = 16.9 pmol g\(^{-1}\) hemoglobin, \(n = 12\)) was 21% higher than the median level of all non-smokers regardless of their diet. The alteration of adduct levels caused by other compounds in cigarette smoke, which are also common food contaminants, is much larger. The levels of the hemoglobin adducts of acrylamide and glycidamide were about 3-fold and about 2.3-fold higher, respectively, in smokers (\(n = 255\)) than those observed in non-smokers (\(n = 255\)) from the European Union.[10] Similarly, Aasa et al.[31] reported that there was a considerable difference between the mean levels of hemoglobin adducts resulting from glycidol exposure between non-smoking (10.3 pmol g\(^{-1}\) hemoglobin, \(n = 6\)) and smoking adults (23.4 pmol g\(^{-1}\) hemoglobin, \(n = 6\)), however, the number of cigarettes per day was not reported. Taken together, despite its presence in the mainstream smoke of cigarettes,[21] smoking seems to contribute less to the overall exposure of furfuryl alcohol compared to respective contributions from that of the other mentioned tobacco smoke components.

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The levels of FFA-Val in newborn blood samples were very low compared to those of the mothers (Figure 3). The median of the quotient between FFA-Val in newborns and in mothers (0.15) was significantly higher for the hemoglobin adducts of acrylamide (0.48), glycidamide (0.38), ethylene oxide (0.43).[32]
and glycidol (0.35).\cite{33} Considering that fetal hemoglobin has only two N-terminal Val instead of four in adults and that the lifespan of fetal erythrocytes (60–90 days) is somewhat shorter compared to that of adult erythrocytes,\cite{34,35} these values led to the conclusion that acrylamide and the three epoxides mentioned cross the placental barrier and cause an internal exposure in the fetuses that is similar to that of the mothers. The low ratio of FFA-Val levels observed in the current study suggests that this may not be the case for furfuryl alcohol. However, the weak positive correlation between the adduct levels determined in maternal and cord blood erythrocytes supported the notion that the internal exposure of the mothers to furfuryl alcohol has a small impact on the FFA-Val levels observed in cord blood samples.

The possible relevance of FFA-Val as a biomarker for the internal exposure to furfuryl alcohol was studied previously in FVB/N mice. Sachse et al.\cite{25} showed that the adduct level increased with the application of 400 mg kg$^{-1}$ body weight furfuryl alcohol. Also, the expression of the human sulfotransferase SUIT1A1/1A2 in a genetically-modified mouse model\cite{24} or the inhibition of the detoxification of furfuryl alcohol by co-exposure to, e.g., ethanol\cite{25} led to increased internal exposure to the sulfate ester and increased FFA-Val levels, indicating that the adduct may be a biomarker of furfuryl alcohol at high exposure levels. However, the current information on FFA-Val does not allow answering the question for the specificity as a biomarker for furfuryl alcohol exposure in case of low exposure. Currently, there is no plausible hypothesis on other substances that may also form the FFA-Val adduct. Endogenous formation, which was hypothesized previously to contribute to the background adduct levels of other heat-induced food contaminants like, e.g., acrylamide\cite{36} and glycidol,\cite{28,37} cannot be ruled out in the case of furfuryl alcohol. This may especially contribute to the low FFA-Val levels observed in the cord blood of newborn children. Future studies on FFA-Val in, e.g., raw food eaters that do not consume heated food stuffs and in humans following controlled diets with defined furfuryl alcohol intake will possibly clarify questions on the sources and the specificity of the FFA-Val adduct as a biomarker of furfuryl alcohol exposure.

In summary, the scattered and incomplete data on furfuryl alcohol contents in food and in other matrices does not allow estimating accurately the furfuryl alcohol uptake in the population. FFA-Val in hemoglobin may be a suitable biomarker for the estimation of the external furfuryl alcohol exposure. The analytical method developed previously\cite{25} allowed quantifying the FFA-Val in two study populations from Belgium and Germany with an overall number of 172 adult participants and adduct levels between 5.7 and 26.3 pmol FFA-Val g$^{-1}$ hemoglobin. It was sufficiently sensitive to also determine FFA-Val in cord blood samples from newborn children with values as low as 0.6 pmol g$^{-1}$ hemoglobin; these adducts levels may result from endogenous furfuryl alcohol exposure and/or from prenatal transfer of furfuryl alcohol via the placenta. In the adult participants, the most obvious source of furfuryl alcohol exposure was tobacco smoking, which, however, did not increase adduct levels to the same extent as observed for adducts from other contaminants that are also present in cigarette smoke. The lower levels of FFA-Val in blood samples from vegans could not be explained by analyzing the correlations between FFA-Val and the food intake reported in the RBVD study. For the future application in reverse dosimetry, we have to determine the increase of FFA-Val with the furfuryl alcohol dose in a controlled exposure study.

4. Experimental Section

Study Populations and Collection of Erythrocytes: The first sample set of erythrocytes of 100 mother–newborn pairs was chosen randomly from the Flemish birth cohort ENVIRONAGE.\cite{26} Pregnant mothers recruited in the East-Limburg Hospital in Belgium between 2013 and 2016 were eligible if they were able to fill out a questionnaire in Dutch, asking for, e.g., age, pregestational body weight and height, or the smoking status. Cord blood was obtained immediately after birth and the maternal blood samples were collected 1 day after birth. The study protocol was approved by the Ethics Committee of Hasselt University and East-Limburg Hospital in Genk, Belgium.

The second sample collection was from the cross-sectional RBVD study with 36 vegans and 36 omnivores (18 men and 18 women each, aged 30–60 years) conducted at the German Federal Institute for Risk Assessment (BfR), Berlin, Germany.\cite{27} The participants recruited in 2017 had followed their current diet for at least 1 year. Dietary habits were documented with a computer-based food frequency questionnaire (FFQ) based on the software EPIC-sof.\cite{28} allowing a validated and standardized assessment of the food intake of 1 year. Anthropometric measures (e.g., weight, height) and the smoking status were assessed and a fasting blood sample was collected. The cross-sectional study (RBVD) was approved by the Ethics Committee of the Charité – Universitätsmedizin Berlin (No. EA4/121/16). Written informed consent was obtained from all participants at the study entry. The ENVIRONAGE study protocol was approved by the Ethics Committee of Hasselt University and East-Limburg Hospital in Genk, Belgium. Written informed consent was obtained from all participating mothers before delivery. Both studies were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Chemicals: Fluorescein-5-isothiocyanate (FITC), purity >90%, formic acid, and HPLC-grade acetone were purchased from Merck (Darmstadt, Germany). Water (UHPLC-MS Optigrade) was purchased from LGC Standards GmbH (Wesel, Germany). All other reagents and solvents (analytical grade) were purchased from Merck. The reference compound 3-fluorescein-1-((furan-2-yl)methyl)-5-(propan-2-yl)-2-thiooxazolidin-4-one (FFA-Val-FTH) and the isotope labeled standards 3-fluorescein-1-((furan-2-yl)methyl)-5-(propan-2-yl)-2-thiooxazolidin-4-one (FFA-Val-d$_{15}$-FTH) were synthesized as described previously.\cite{25} Edman Degradation of the N-Terminal Val in Hemoglobin and Solid-Phase Extraction of FFA-Val-FTH: Erythrocytes were isolated by centrifugation of EDTA blood samples (~ 8 mL) at 3500xg for 10 min at room temperature. The pellet was washed twice with 0.9% aqueous sodium chloride (4 mL), lyzed by adding 4 mL of water and stored at ~80 °C. The hemoglobin content determined with a HemoCue Hb 201+ analyzer (Radiometer, Willich, Germany) varied from 10.4 to 15.5 g hemoglobin dL$^{-1}$. The technique for the quantification of FFA-Val after a FITC-mediated Edman degradation for the cleavage of the adduct was described previously.\cite{25} Briefly, 250 μL aliquots of the reconstituted erythrocytes were mixed with 15 μL of 1 M potassium hydrogen carbonate and 20 μL of a solution of FFA-d$_{15}$-Val-FTH (37.73 nm). The Edman reaction was started by adding 30 μL of 428 mM FITC in N,N-dimethylformamide. The samples were incubated at 37 °C and mixed at 800 rpm for 16 h. Cell debris and protein was precipitated by addition of 1.6 M acetonitrile. After thorough mixing of the samples and centrifugation (15000 g, 10 min), the pH of the supernatants was adjusted by adding 25 μL of 1 M aqueous ammonium hydroxide. The analytes were enriched on mixed-mode anion exchange cartridges Oasis MAX (60 mg; Waters, Eschborn, Germany). After preconditioning with 2 mL of acetonitrile and 2 mL of water, the samples were loaded and the columns were washed with 2 mL each of acetonitrile, water, and 0.5%
aqueous cyanocetic acid. The analytes were eluted with 1.6 mL of 0.25% cyanocetic acid dissolved in acetonitrile/water (6:4). The solvents were evaporated under reduced pressure and the samples were reconstituted in 50 μL of water/acetonitrile (1:1).

UHPLC-MS/MS of FFA-Val-FTH: The residuals from solid-phase extraction were subjected to reversed-phase chromatography using an Acquity UPLC system (Waters). The analytes were detected with a QTrap6500 (Sciex, Darmstadt, Germany) with an electrospray ionization source operating in the positive ionization mode. Samples of 8 μL were injected onto a Hypersil GOLD column (1.9 μm, 2.1 × 150 mm; Thermo Scientific, Dreieich, Germany), applying a gradient program with the solvents A (water + 0.1% fornic acid) and B (acetonitrile + 0.1% formic acid) and a flow rate of 0.35 mL min⁻¹. The gradient was: 0–1 min (90% A), 1–16 min (90–40% A), 16–17.5 min (0% A), 17.5–19 min (90% A). The analyte FFA-Val-FTH and the reference substance FFA-d7-Val-FTH were monitored by multiple reaction monitoring (MRM). Two characteristic fragmentation reactions of the analyte FFA-Val-FTH were monitored in parallel to the corresponding transitions of the isotope-labeled standard FFA-d7-Val-FTH. The peak area of the signal reflecting the concomitant loss of the isopropyl- and the 2-methylfuran moieties of the analyte (m/z 569.1 → 390.1; collision energy, 70 V; cell exit potential, 20 V) was used as the quantifier signal. The slightly lower peak caused by abstraction of the 2-methylfuran cation (m/z 569.1 → 810; collision energy, 100 V; cell exit potential, 10 V) was used as qualifier signal. The other MS-parameters were as follows: curtain gas, 20 psi; collision activated dissociation (CAD) gas, medium; ion source temperature, 450 °C; ion spray voltage, 5500 V; ion source gas 1, 60 psi; ion source gas 2, 50 psi. The data was recorded and analyzed with Analyst 1.6.2 Software (Sciex).

Calculation of FFA-Val Levels in Hemoglobin Samples: The levels of FFA-Val (A) in the hemoglobin samples (pmol g⁻¹ hemoglobin) of study participants were calculated as follows:

\[
A = \frac{p_{\text{total}} \times \text{amount}_{\text{IS}} \times \text{cf}}{\text{amount}_{\text{Hb}}} \times \text{cf}
\]

with the peak areas of the quantifier signals of the analyte FFA-Val-FTH (\(p_{\text{total}}\), m/z 569.1 → 390.1) and of the internal standard FFA-d7-Val-FTH (\(p_{\text{IS}}\), m/z 576.1 → 390.1), with the quantities of the internal standard (\(\text{amount}_{\text{IS}}\)) and of hemoglobin (\(\text{amount}_{\text{Hb}}\)) used for the Edman degradation, and a conversion factor (cf) reflecting the yield of the Edman degradation. The factor was determined independently for each daily batch of 24–48 samples. Three quality control samples were added to the batch and in addition three quality control samples containing 1 pmol of the peptide FFA-Val-Leu. After the cleavage reaction, the conversion factor was calculated from the amount of FFA-Val-FTH in the spiked samples with FFA-Val-Leu after subtraction of the background. The mean Edman degradation yield (± standard deviation) determined on 16 different days was 82.0 ± 13.8%.

Statistics: Statistical analyses were conducted with SAS version 9.4. The adduct levels in blood samples were presented as median and interquartile range (IQR) and the differences were analyzed with the Mann-Whitney rank-sum test. Differences with p-values < 0.05 were considered statistically significant. The coefficients of the correlations between the FFA-Val levels in cord blood and maternal blood (ENVIRONAGE) and between FFA-Val levels and intake of different food groups (derived from a FFQ in the RBVD study) were determined with the Spearman function.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
B.H.M.: conceptualization, methodology and investigation, project administration, visualization, writing - original draft; N.B.: methodology and investigation, writing - review & editing; J.G.F.H.: data curation, writing - review & editing, formal analysis; T.S.N.: conceptualization, funding acquisition; I.T.: data curation, writing - review & editing; K.A.: conceptualization, writing - review & editing.

Data Availability Statement
The datasets generated and/or analyzed during the current RBVD study and the ENVIRONAGE study are not publicly available due to provisions of the written informed consent.

Keywords
birth cohort, coffee, cord blood, furfuryl alcohol, hemoglobin adducts, vegan diet

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