



Contents lists available at ScienceDirect

Environment International

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Endotoxin levels in cow's milk samples from farming and non-farming families – The PASTURE study

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ARTICLE INFO

Article history:

Received 16 October 2007

Accepted 9 April 2008

Available online xxxx

Keywords:

Allergy
Endotoxin
Farming
Milk

ABSTRACT

Background: Children from farming families have less allergies than their peers. Consumption of farm milk or unpasteurized milk has been shown to explain (part of) the farming effect or protect against allergies independent of farming status.

Objectives: We investigated whether the protective effect of farm milk consumption can be explained by higher levels of bacterial endotoxin in milk.

Methods: We measured endotoxin in approximately 400 farm milk and shop milk samples from farming and non-farming families, respectively, with the kinetic chromogenic Limulus Amebocyte Lysate test and compared endotoxin levels between groups defined by farming status and type of milk (farm milk/shop milk).

Results: Endotoxin levels were significantly higher in milk samples from non-farming families compared to farming families [adjusted geometric means ratio (95% confidence interval)=2.61 (1.53–4.43)]. No significant difference in endotoxin levels was found between shop milk and farm milk samples [adjusted geometric means ratio (95% confidence interval)=1.56 (0.94–2.58)]. The difference between farming and non-farming families could be explained completely for farm milk and partially for shop milk by storage conditions and temperature during transportation to the fieldworker's home.

Conclusion: The farming effect and the effect of farm milk consumption cannot be explained by higher levels of endotoxin in milk from farmers and farm milk, respectively.

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1. Introduction

Children from farming families have been shown to have a decreased risk of becoming atopic and having symptoms of hay

fever and asthma (Braun-Fahrlander et al., 1999; Ernst and Cormier, 2000; Kilpelainen et al., 2000; Klintberg et al., 2001; Riedler et al., 2000; Von Ehrenstein et al., 2000). Consumption of farm milk (Ege et al., 2006; Riedler et al., 2001) or unpasteurized milk (Perkin and Strachan, 2006; Wickens et al., 2002) has been shown to explain (part of) the farming effect or protect against allergies independent of farming status.

The mechanism behind the protective effect of the consumption of farm milk or unpasteurized milk, however, is unclear. Cook et al. (2000) and Radon et al. (2004) found an association between consumption of unpasteurized milk and seropositivity to *Toxoplasma gondii*. Seropositivity to *T. gondii* has been found to be associated with a decreased risk of asthma and allergies in some studies (Linneberg et al., 2003; Matricardi et al., 2000; Matricardi et al., 2002), but not in the study by Radon et al. (2004). Another explanation for the farm

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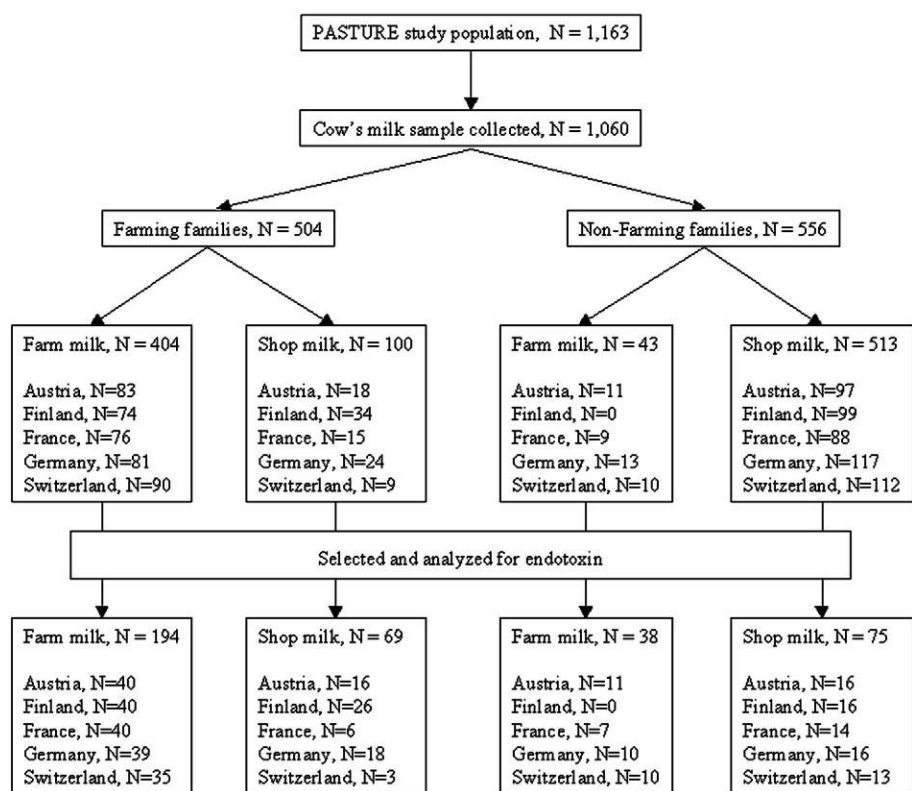


Fig. 1. Schematic overview of the study population, number of cow's milk samples collected and analyzed for endotoxin in relation to farming status and type of milk.

milk effect might be the presence of higher levels of non-infectious microbial agents in unpasteurized farm milk compared to pasteurized milk. Unpasteurized milk is rich in gram-negative bacteria (Kilewein,

1994) and their lipopolysaccharides (Suhren et al., 1986). Levels of endotoxins, cell wall components of the outer membrane of gram-negative bacteria, have been found to be increased in house dust of

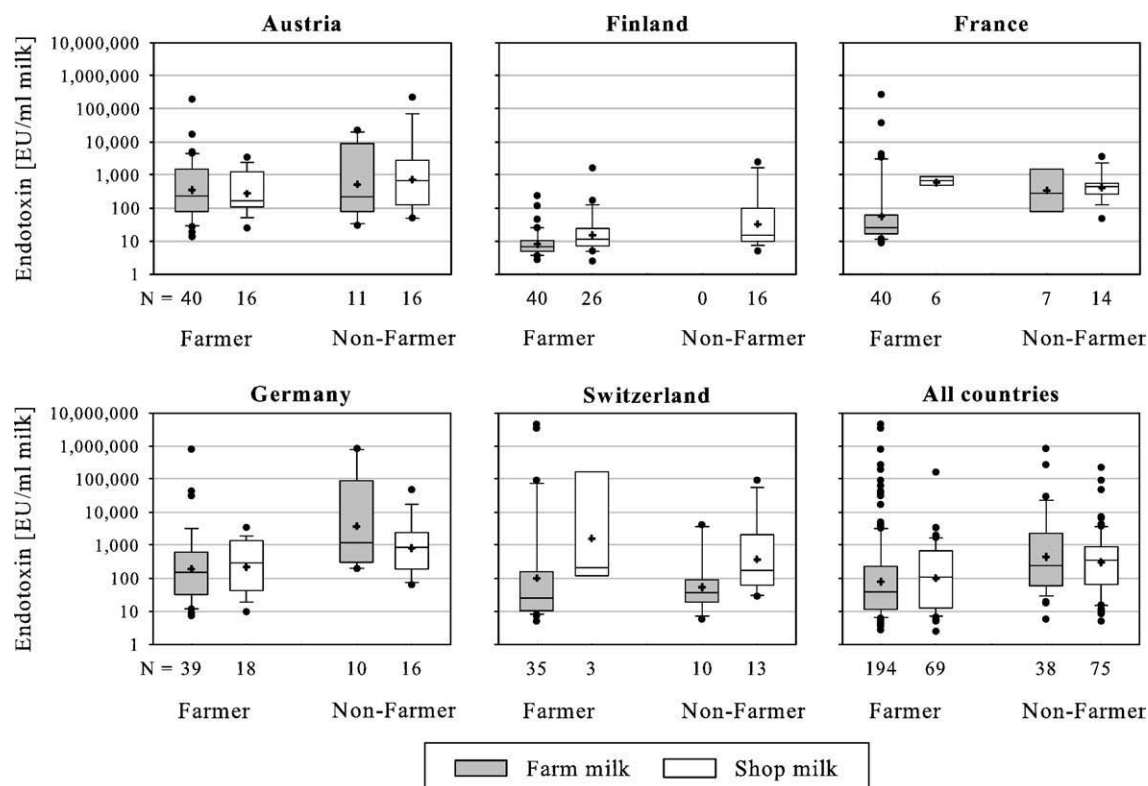


Fig. 2. Box-plots of endotoxin levels [EU/ml] in milk samples in relation to farming status and type of milk. Box-plots show medians, 10th, 25th, 75th, and 90th percentiles; the dots are the values outside the 10th and 90th percentiles of the distributions. Plusses represent geometric means.

farmers compared to non-farmers (von Mutius et al., 2000) and to be associated with lower prevalence of atopy, atopic asthma, and hay fever (Braun-Fahrlander et al., 2002; Gehring et al., 2002; Gereda et al., 2000). It is not clear, whether the ingestion of endotoxin has an effect on the development of asthma and allergies. However, it has been shown that the oral administration of certain probiotics, i.e. of cultures of potentially beneficial bacteria of the gut microflora, may reduce the risk of atopic dermatitis (Kalliomaki et al., 2001).

Within the collaborative PASTURE study (Protection against allergy: study in rural environments) we measured and compared levels of bacterial endotoxin in four groups of cow's milk samples: farm milk from farming and non-farming families, and regular milk bought in a shop (in the following referred to as "shop milk") from farming and non-farming families, respectively.

2. Methods

2.1. The PASTURE study

Data were collected in the framework of the PASTURE study, a prospective birth cohort study located in rural areas of five European countries: Region south of Munich, Southern Germany; Salzburg area, North-Western Austria; North-Eastern part of Switzerland; Kuopio, Jyväskylä and Joensuu areas, Central Finland; and Franche-Comté, Eastern France. The overall aim of the study is the assessment of the role of exposure to various microbial products for the development of childhood asthma and allergies in a prospective design in rural environments across Europe. Between August 2002 and March 2005, in each country around 100 pregnant women living on a farm (or running a farm) where livestock is held and 100 pregnant women from the same area not living on a farm were recruited. The study has been approved by the local ethic committees and was carried out in accordance with the institutional guidelines for the protection of human subjects. Written informed consent was obtained from all participants.

2.2. Cow's milk samples

When the child was approximately two months old, a home visit was performed. During the home visit samples from the milk that is usually consumed in the family were collected following a standardized protocol. In brief, 4 samples of 50 ml were filled into sterile tubes using a pipette or syringe. If possible, milk samples were taken from the vessel in which the family usually keeps the milk for their own consumption. In case the milk was not kept in any household vessel, but in the farm's tank (farm milk) or in the package in which it was bought (shop milk), it was taken from the farm's tank or decanted directly into the tube, respectively. If there was no package open, the one to be consumed next was opened for sampling purposes upon approval of the family. A field form concerning information relevant to the collection of the milk sample as well as potential determinants of endotoxin levels in milk such as storage location, duration, and temperature; and boiling of the milk (farm milk only) was completed. After transportation to the fieldworker's home, samples were frozen at least -18°C .

For the present project due to financial constraints not more than 400 milk samples (80 samples from every center) could be analyzed for endotoxin. Four groups of samples were defined by farming status of the family and type of milk (farm milk/shop milk). In every country, samples were selected as follows: approximately 40 samples were randomly selected from the farmers' farm milk samples. In addition, all farm milk samples from non-farmers' were selected. The remaining samples were selected such that half of them were a random sample of shop milk samples from farmers and non-farmers, respectively. A schematic overview of the number of cow's milk samples that have been collected and analyzed is given in Fig. 1.

Table 1

Mutually adjusted geometric means (GM) and geometric means ratios (GMR) with 95% confidence intervals (CI) for the association between endotoxin levels in milk samples and farming status, type of milk, and country

	GM	GMR	(95% CI)	p-value
Farmer				
Yes	260		Reference	
No	100	2.61	(1.53–4.43)	0.0004
Type of milk				
Farm milk	129		Reference	
Shop milk	201	1.56	(0.94–2.58)	0.0844
Country				
Austria	476		Reference	
Finland	17	0.03	(0.02–0.07)	<0.0001
France	163	0.33	(0.16–0.66)	0.0020
Germany	459	0.92	(0.48–1.79)	0.8171
Switzerland	169	0.34	(0.17–0.70)	0.0038

Table 2

Distribution of factors that might explain differences between farming and non-farming families for farm milk and shop milk samples, respectively

	Farm milk				Shop milk				
	Farmer (N=154)		Non-farmer (N=38)		Farmer (N=69)		Non-farmer (N=75)		
	n/ N ^a	%	n/N ^a	%	n/N ^a	%	n/N ^a	%	
<i>Factors relating to the time before sample collection</i>									
Number of hours stored since taken from tank									
≤4 h	37/139	26.6	3/31	9.7	–	–	–	–	
4–12 h	57/139	41.0	5/31	16.1	–	–	–	–	
12–24 h	24/139	17.3	14/31	45.2	–	–	–	–	
>24 h	21/139	15.1	9/31	29.0	–	–	–	–	
Milk was stored in refrigerator									
Yes	114/153	74.5	35/38	92.1	66/69	95.6	68/74	91.9	
No	39/153	25.5	3/38	7.9	3/69	4.4	6/74	8.1	
Milk was boiled									
Yes	19/154	12.3	4/34	11.8	–	–	–	–	
No	135/154	87.7	30/34	88.2	–	–	–	–	
Number of days stored since pack was opened									
0 days	–	–	–	–	31/66	47.0	33/74	44.6	
0–1 day	–	–	–	–	27/66	40.9	25/74	33.8	
>1 day	–	–	–	–	8/66	12.1	16/74	21.6	
Percentage fat									
≤2%	–	–	–	–	36/67	53.7	32/72	44.4	
>2%	–	–	–	–	31/67	46.3	40/72	55.6	
<i>Factors relating to the time after sample collection</i>									
Temperature during transportation to fieldworker's home									
≤4 °C	93/122	76.2	22/31	71.0	25/57	43.9	41/62	66.1	
>4 °C	29/122	23.8	9/31	29.0	32/57	56.1	21/62	33.9	

All countries combined, but Finland was excluded for farm milk since there were no farm milk samples from Finnish non-farming families.

^a In some cases due to missing values, N is smaller than the total number of samples analyzed given above.

2.3. Analysis of endotoxin in milk samples

First, milk was diluted with pyrogen-free water. The standard dilution was 1:500, the maximum dilution was 1:1000000. From the final dilution, an aliquot of 100 µl was added to a microtiter plate. Endotoxin was analyzed with the kinetic chromogenic Limulus Amebocyte Lysate (LAL) test, using the same batch of LAL reagents and standards for all analyses (BioWhittaker, Walkersville, MD, USA; LAL lysate lot no. 3L433E, lipopolysaccharide (LPS) standard lot no. 3L2950) (Douwes et al., 1995). The amount of endotoxin in a sample was expressed as endotoxin units (EU) per milliliter of milk. All endotoxin levels were above the limit of detection of the assay of 5 EU/ml.

2.4. Statistical methods

Levels of endotoxin were approximately normally distributed after natural log (ln)-transformation. Multiple linear regression analyses based on ln-transformed endotoxin levels were performed to compare endotoxin levels between groups defined by farming status and type of milk. Simple and multiple linear regression analyses based on ln-transformed endotoxin levels were performed to assess the impact storage conditions and other sample characteristics on endotoxin levels. Results are presented as adjusted geometric means ratios (GMR) calculated from regression coefficients β as $\text{GMR} = \exp(\beta)$ with 95% confidence intervals (CI), i.e. as the ratio of the geometric mean in the exposed group and the geometric mean in the reference category. Statistical significance was defined by a two-sided α -level of 5%. Calculations were performed using the Statistical Analysis System (SAS, version 9.1, Cary, NC, USA).

3. Results

3.1. Levels of endotoxin in milk samples

The country-specific and overall number of endotoxin measurements in milk samples together with the distribution of endotoxin levels in relation to the family's farming status and type of milk are presented in Fig. 2. Endotoxin levels varied between countries. Levels were much lower in Finland compared to the other countries.

3.2. Associations of endotoxin levels with farming status and type of milk

With few exceptions, endotoxin levels in milk samples of non-farming families were somewhat higher than endotoxin levels in milk samples of farming families; and shop milk samples contained more endotoxin than farm milk samples. Due to the small

numbers of samples per country (except for farm milk samples from farming families), subsequently comparisons between farmers and non-farmers and between farm milk and shop milk were made for all countries combined with adjustment for country to account for differences between countries. Results are presented in Table 1. Across all countries, endotoxin levels were significantly higher in milk samples from non-farming families compared to farming families, whereas no statistically significant difference was found between farm milk and shop milk. There was no significant interaction between farming status of the family and type of milk (data not shown).

3.3. Factors explaining differences between farming and non-farming families

Frequency distributions of factors related to storage and treatment of the milk before and after sampling and that might explain differences in endotoxin levels between farming and non-farming families at the time of consumption and laboratory analysis, respectively, are presented in Table 2. Factors can be divided into factors relating to the time *before* sample collection, which may have an impact on endotoxin levels at the time of consumption and factors relating to the time *after* sample collection, which may have an impact on endotoxin levels at the time of laboratory analysis. Farm milk had been stored for significantly longer periods after being taken from the farm's tank by non-farming families compared to farming families. Furthermore, it had been stored in the refrigerator significantly more often by non-farming families compared to farming families. Temperature during transportation from the family's home to the fieldworker's home was significantly higher for milk samples from farming families compared to non-farming families for unknown reasons. None of the other factors was significantly associated with the family's farming status or type of milk.

Results from linear regression analyses comparing farming and non-farming families by type of milk taking into account country and storage and treatment of milk are presented in Table 3 for farm and shop milk, respectively. Longer storage times of farm milk and higher temperatures during transportation were associated with significantly higher levels of endotoxin. Keeping the milk outside the refrigerator and not boiling farm milk were also associated with higher levels of endotoxin. However, these associations were not statistically significant. Differences in endotoxin levels between farm milk samples of farming and non-farming families could be explained by differences in storage time and temperature during transportation. Differences in endotoxin levels between shop milk samples of farming and non-farming families became somewhat smaller and statistically non-significant taking into account storage and treatment of the milk.

Table 3

Adjusted geometric means ratios (GMR) and 95% confidence intervals (CI) for the association between endotoxin levels in milk samples and farming for farm milk and shop milk from multiple linear regression analyses with and without taking into account storage and treatment of samples

Variable	Farm milk		Shop milk	
	Adj. GMR (95% CI)	p-value	Adj. GMR (95% CI)	p-value
<i>Adjusted for country only</i>				
Farmer				
No vs yes	2.97 (1.18–7.47)	0.0215	1.92 (1.05–3.50)	0.0364
<i>Additionally adjusted for storage and treatment of samples</i>				
Farmer				
No vs yes	1.07 (0.30–3.82)	0.9139	1.62 (0.79–3.31)	0.1871
Country				
Finland vs Austria	–	–	0.01 (0.00–0.06)	<0.0001
France vs Austria	0.10 (0.01–0.75)	0.0262	0.41 (0.08–2.15)	0.2914
Germany vs Austria	0.30 (0.03–2.83)	0.2964	0.25 (0.06–1.14)	0.0762
Switzerland vs Austria	0.12 (0.02–0.84)	0.0352	0.77 (0.17–3.41)	0.7313
Milk was stored in refrigerator				
No vs yes	1.81 (0.49–6.64)	0.3731	2.37 (0.52–10.83)	0.2682
Number of hours since taken from tank				
4–12 h vs ≤4 h	1.91 (0.56–6.49)	0.3039	–	–
12–24 h vs ≤4 h	7.72 (1.72–32.00)	0.0081	–	–
>24 h vs ≤4 h	8.20 (1.63–41.22)	0.0118	–	–
Milk was boiled				
No vs yes	1.87 (0.46–7.57)	0.3811	–	–
Number of days since pack was opened				
0–1 day vs 0 days	–	–	1.05 (0.49–2.26)	0.8928
>1 day vs 0 days	–	–	0.72 (0.24–2.19)	0.5635
Percentage fat				
>2% vs ≤2%	–	–	0.68 (0.24–1.95)	0.4791
Temperature during transportation to fieldworker's home				
>4 °C vs ≤4 °C	5.21 (1.02–26.49)	0.0489	2.28 (0.94–5.56)	0.0720

All countries combined, but Finland was excluded for farm milk since there were no farm milk samples from Finnish non-farmers.

4. Discussion

Endotoxin levels in farm and shop milk samples were significantly higher for non-farming families compared to farming families. The difference could be explained completely for farm milk and partially for shop milk by storage conditions and temperature during transportation to the fieldworker's home. No significant difference in endotoxin levels was found between farm and shop milk samples.

4.1. Differences between countries

Endotoxin levels in farm and shop milk samples differed between countries (Table 1). For both, farm milk and shop milk, highest levels were measured in samples from Austria and lowest levels were measured in samples from Finland (Table 3). Differences between countries remained after adjustment for sample characteristics. The reason for this is not clear. All samples have been analyzed in a standardized manner in the same laboratory. However, bacterial microorganisms used to produce cheese and other fermented milk products are product-specific contributing to texture and taste (Beresford et al., 2001). The types of cheese and other fermented milk products that are kept in the refrigerator of the participating families (in most cases together with the milk) and consequently the type of bacteria present most likely differ between countries, which may result in differences in endotoxin levels. In addition, bacterial contamination by other foods stored in the refrigerator may have an impact on milk endotoxin levels and contribute to differences between countries.

4.2. Differences between farming and non-farming families

The results of the present study do not support our hypothesis that farm milk contains higher levels of endotoxin compared to shop milk or that milk from farming families contains more endotoxin than milk from non-farming families. In contrast, farm and shop milk samples from non-farming families contained 3- respectively 2-times more endotoxin than farm and shop milk samples from farming families. For farm milk, this difference is in large part explained by the fact that non-farming families keep the milk for longer periods before consumption. This is plausible, since non-farming families have to buy their milk somewhere on a farm at regular intervals, whereas the majority of the farming families that drinks farm milk (82%) have their own dairy cows and consumes the milk more or less immediately after it has been taken from the farm's tank. Furthermore, farming families may consume more milk than non-farming families and therefore store milk for shorter periods. In addition, farmers keeping dairy cows have to be more aware of the importance of good hygienic conditions for the quality of the milk. This may also have changed over time so that findings from this study, based on recent milk samples, may not be unconditionally applicable to findings from e.g. the ALEX study, which was on school children whose exposure to farm milk in early life is now already more than 15 years ago. For shop milk samples we can only speculate about the reasons for the difference between farmers and non-farmers. Hygienic conditions may be different in farming and non-farming families. Furthermore, farming families may differ from non-farming families with regard to the contents of their refrigerator; i.e. farming families may keep a different assortment of food in their refrigerator compared to non-farming families or keep certain types of food less long in the refrigerator before consumption since they are (freshly) available from their own farm. No information on these factors is available from the present study. However, endotoxin levels in farm milk samples were positively associated with the time since the milk has been taken from the farm's tank, while the number of days since the pack was opened had no impact on endotoxin levels in shop milk samples (Table 3). This indicates that this argument may hold for farm milk but probably not for shop milk. Future studies including questions on hygienic conditions in the

refrigerator and storage of fresh foods in the refrigerator are needed to investigate this issue in more detail. There is some indication that the magnitude of these differences may differ between countries, however, except for farm milk samples from farming families, the number of samples per country is too small to yield reliable country-specific estimates. Besides these factors relating to the time *before* sample collection and which have an impact on endotoxin levels in milk as consumed by the families, we found temperature during transportation to the fieldworker's home to be a determinant of milk endotoxin levels explaining part of the difference between farming and non-farming families at the time of laboratory analysis. The reason for this difference is not clear. We tried to standardize treatment of samples as much as possible. However, for some reason we did not succeed completely. This means that the difference in milk endotoxin levels between farming and non-farming families at the time of consumption is probably less pronounced than the difference we find at the time of laboratory analysis. However, the present results still argue against higher endotoxin levels in milk of farming families compared to non-farming families.

5. Conclusion

We conclude from our results that the farming effect and the protective effect of farm milk consumption cannot be explained by higher levels of endotoxin in milk from farming families compared to non-farming families and farm milk compared to shop milk, respectively.

Acknowledgements

We wish to thank the collaborators of the PASTURE study teams. The PASTURE study was funded within the European Union "Quality of Life and Management of Living Resources" program, key action "Environment and Health", proposal no. QLRT-2001-00250. Ulrike Gehring was supported by a post-doc fellowship of the German Academic Exchange Service (DAAD) and a VENI research fellowship of the Netherlands Organization for Scientific Research (NWO).

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