doi: 10.1111/j.1365-2222.2008.03008.x

Clinical and Experimental Allergy, **38**, 1342–1348

ORIGINAL PAPER Clinical Allergy

Impact of maternal atopy and probiotic supplementation during pregnancy on infant sensitization: a double-blind placebo-controlled study

A. Huurre*, K. Laitinen*†, S. Rautava*, M. Korkeamäki‡ and E. Isolauri*

**Department of Pediatrics, Turku University Central Hospital and University of Turku, Turku, Finland, †Department of Biochemistry and Food Chemistry and Functional Foods Forum, University of Turku, Turku, Finland and ‡CyFlo, Turku, Finland*

Clinical and Experimental Allergy

Summary

Background The effects of breastfeeding and probiotics on infant sensitization still remain discrepant.

Objective To explore probable explanatory factors in infant sensitization and the protective effect of probiotics.

Methods Altogether 171 mother–infant pairs from an ongoing placebo-controlled double-blind study with nutrition modulation by dietary counselling and probiotic supplementation were studied. Skin prick testing was done in infants at 6 and 12 months and in mothers at third trimester of pregnancy. The breast milk concentrations of cytokines TGF-β2, soluble CD14, IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were measured.

Results The risk of sensitization increased in infants with allergic mothers breastfeeding over 6 months [odds ratio (OR = 4.83, $P = 0.005$)], or exclusively breastfeeding over 2.5 months (OR = 3.4, $P = 0.018$). Probiotic supplementation had a protective effect against sensitization in infants with a high hereditary risk due to maternal sensitization (OR = 0.3, $P = 0.023$). The concentration of TGF-β2 tended to be higher in the colostrum of the mothers in the probiotic group as compared with those on placebo (probiotic/placebo ratio = 1.50, $P = 0.073$). A similar result was obtained in the subgroup of allergic mothers (probiotic/placebo ratio = 1.56, $P = 0.094$).

Conclusion Infants of atopic mothers, specifically when breastfed exclusively over 2.5 months or totally over 6 months, had a higher risk of sensitization at the age of 12 months. This risk could be reduced by the use of probiotics during pregnancy and lactation, partly by resulting in a beneficial composition of the breast milk.

Correspondence:
Anu Huurre, Department of Pediatrics, Turku University Central Hospital and University of Turku, Kiinanmyllynkatu 4-8, 20520 Turku, Finland.
E-mail: anu.huurre@utu.fi

Keywords breast milk cytokines, breastfeeding, probiotics, sensitization
Submitted 10 August 2007; revised 6 November 2007 and 26 February 2008; accepted 4 March 2008.

Introduction

Human milk provides an optimal source of nutrition, simultaneously guiding the maturational processes in infancy [1, 2]. Breastfeeding, moreover, remains the golden standard in allergy prevention strategies. The preventive effect is assumed to culminate in infants with atopic heredity [3]. Epidemiological evidence thus far suggests, however, that breastfeeding may both reduce [3–5] and increase [6, 7] the risk of atopic diseases in such infants. It is in fact not rare for infants at risk to manifest allergic symptoms during exclusive breastfeeding [8]. One possible reason for this may lie in the components of breast milk, and we have recently reported that allergic mothers had lower concentrations of TGF-β2 in their breast milk [9], and that this could be compensated for by the use of probiotics [10].

The hygiene hypothesis originated from epidemiological findings, demonstrating that children growing in smaller families were at increased risk of developing atopic disease, possibly attributable to a lower incidence of infections in early childhood [11]. On the basis of such a conception a gut microbiota hypothesis has recently been proposed [12], suggesting that alterations in the gut microbiota, the early and most massive source of microbial exposure, may underlie the atopic epidemic. In this perspective, the use of probiotic supplementations seems attractive. Indeed, preliminary data suggest that specific probiotics, if supplemented perinatally, may reduce the risk of atopic eczema in the child [13–15]. However, the

evidence for a probiotic potential against immunological sensitization is insufficient. The specific probiotic strains successful in the prevention of infectious diseases, food allergy or atopic eczema may not target sensitization [13], or the population studied may not have been responsive to the immune modulation [16]. These considerations underline the importance of pre-clinical testing and selection of strains, with confirmation of their viability to exert any anti-inflammatory influence [17, 18], as well as of initiating probiotic intervention in the pre-natal period in an attempt to reduce the risk of disease in the child. It is likewise important to examine the selection of study populations and possible foods combined in probiotic intervention, because probiotic effects also depend on other components in the diet [19].

In the present placebo-controlled prospective intervention study, we assessed the role of maternal atopic status on sensitization of the infant, and also as a potential target of allergy prevention with probiotics. To explore probable explanatory factors, the effect of maternal allergic status on the cytokine profile of breast milk, and thereby on infant sensitization, was measured.

Materials and methods

Subjects and study design

Altogether 171 mother–infant pairs from an ongoing placebo-controlled double-blind study with nutrition modulation by dietary counselling and probiotic supplementation were studied (described in detail in [20]). The enrolment criteria were identical diet counselling in allergic families (i.e. dietary counselling groups of the original study), no chronic or metabolic disease in the mother before or during early pregnancy and completed follow-up and skin prick test (SPT) of the infant at the age of 1 year (140 infants). To have a homogenous group of mothers, only the mothers in the nutrition-counselling group receiving exactly the same nutritional advice during pregnancy and breastfeeding were included, as the diet of the pregnant and lactating mother may affect the risk of atopic disease. The mothers were randomized to receive either probiotics, that is both *Lactobacillus rhamnosus* strain GG (ATCC 53103, Valio Ltd, Helsinki, Finland) and *Bifidobacterium lactis* Bb12 (Chr. Hansen, Hørsholm, Denmark) 1×10^{10} CFU/day each ($n = 72$), or placebo (microcrystalline cellulose and dextrose anhydrate, Chr. Hansen) ($n = 68$) from the first trimester of pregnancy to the end of exclusive breastfeeding. The study was approved by the Ethical Committee of the Hospital District of South-West Finland. Informed consent was obtained from the mothers. The study is registered (http:/www.clinicaltrials.gov/ct/gui/show/NCT00167700). Atopic sensitization of the infants was assessed by SPT at the ages of 6 and 12 months and in mothers at third trimester of pregnancy as described previously [21]. The antigens tested included cow's milk, egg white, wheat and rice flour both diluted 1/10 (w/v) with 0.9% (w/v) sodium chloride, gliadin diluted 1 mg/mL with an ethanol/glyceroleum/ALK-diluent (Allergologisk Laboratorium A/S, Hørsholm, Denmark) mixture, cod, soya bean, birch, six grasses, cat, dog, *Dermatophagoides pteronyssimus* allergen Der p1 (Allergologisk Laboratorium A/S), latex (Starallergens S.A., Anthony Cedex, France) and potato, carrot and banana by prick–prick technique, and for mothers we also tested peanut, hazelnut, alder and mugwort (Allergologisk Laboratorium A/S).

Mothers visited the study clinic three times before delivery and mothers and infants at infants' ages of 1, 6 and 12 months. The infants were clinically examined in blinded fashion by the study nurse at 1 month of age and by the physician at 6 and 12 months of age. Atopic eczema was diagnosed according to the criteria introduced by Hanifin [22] in the case of a positive SPT to comply with current definition [23].

Determination of cytokine concentrations in breast milk

Breast milk samples were collected immediately after birth in the maternity hospital and 1 month after delivery, and frozen for later analysis. The concentrations of TGF-β2 and soluble CD14 (sCD14) were measured from whey using commercial sandwich ELISAs specific for these molecules (R&D Systems Europe Ltd, Abingdon, UK). Before analysis the milk samples were thawed and centrifuged at 10 000 g for 10 min to separate cells and fat from whey.

The concentrations of cytokines IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were measured from whey through a multiplexed flow cytometric assay using a commercial Human T-helper types 1 and 2 (Th1/Th2) Cytokine Kit (BD Immunocytomery Systems, Dendermonde, Belgium). Before analysis 12 mmol/L sodium taurocholate synthesized from cholic acid (Sigma-Aldrich Inc., Basel, Switzerland) was added to an equal volume of milk samples, and these were incubated for 30 min before being centrifuged at 12 000 g. Previous experience [24, 25] and our preliminary recovery measurements suggested implementation of this procedure to improve the recovery of the cytokines.

Statistical methods

Infant sensitization assessed by SPT at the age of 12 months was the primary variable. The effect of probiotic intervention, the allergy status of mother and the duration of total and exclusive breastfeeding on infant sensitization were analysed using the χ^2 test. The duration of total breastfeeding and exclusive breastfeeding were divided into two categories, < 6 months vs. $\geqslant 6$ months and < 2.5 months vs. $\geqslant 2.5$ months, respectively. In order to examine whether the effect of breastfeeding on infant

sensitization was different among infants with or without hereditary risk due to maternal allergy, the interaction between maternal atopy status and the duration of breastfeeding was tested by the Mantel–Haenszel method. The homogeneity of the odds ratios (ORs) was tested by the Breslow–Day test. The same method was used to study whether the effect of probiotic supplementation depended on the allergy status of the mother. The results are given as ORs with 95% confidence intervals (CIs).

The cytokine concentrations in breast milk measured immediately after delivery and 1 month later were the secondary variables. The *t*-test for independent samples was used to compare the probiotic group with the placebo group. The cytokine distributions were skewed to the right and were logarithmically (ln) transformed before analysis. The results are given as geometric means, and because of logarithmic transformation, the group comparisons are given as ratios probiotic/placebo with 95% CI. The cytokines were divided into tertiles (T1, T2 and T3), and the association between infant sensitization was given descriptively.

The baseline and clinical characteristics were analysed using the χ^2 test, the *t*-test for independent samples or the Mann–Whitney *U*-test. Data were analysed with SPSS (version 14.0; SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics

The characteristics of mothers, infants and the delivery mode are given in Table 1.

Table 1. Clinical characteristics

	Probiotic group ($N = 72$)	Placebo group ($N = 68$)
Maternal allergic disease (%)	80.6	77.9
Maternal SPT reactivity (%)	65.3	50.0
Gestational age (weeks)	39.9 (1.3, 36.9–42.1)	40.1 (1.3, 35.6–42.3)
Caesarean section (%)	18.2	16.7
Birth weight (g)*	3486 (415, 2250–4470)	3656 (358, 2950–4400)
Birth length (cm)*	50.8 (1.8, 46.0–55.0)	51.4 (1.6, 48.0–55.0)
Exclusive breastfeeding (months)	4.0 (2.5–4.5)	4.0 (2.5–5.0)
Total breastfeeding (months)	8.0 (5.0–10.5)	9.0 (5.0–12.0)

Results are given as percentages, as means (SD, min–max) or as medians (inter-quartile range)

*$P < 0.05$. No statistically significant differences in other variables.

SPT, skin prick test.

Sensitization of the infants

At the age of 12 months, 30% of the infants showed one or more positive reactions in the SPT. The most common allergens responsible for positive reactions were egg white (26% of all infants) and cow's milk (7% of all infants). Allergic disease and positive SPT in the mothers were likely to increase the risk of sensitization of the child: altogether 21% of the infants ($n = 6/29$) of healthy mothers evinced positive SPT reactions at the age of 12 months as compared with 23% of the infants ($n = 8/35$) whose mothers had allergic disease but no SPT reactivity (OR = 1.14, 95% CI 0.34–3.75, $P = 0.835$) and 37% of those ($n = 28/76$) whose mothers had allergic disease and positive SPT (OR = 2.24, 95% CI 0.81–6.15, $P = 0.119$). In the same vein, a positive SPT in the mother, whether or not with allergic disease, tended to increase the risk of positive SPT in the infant (OR = 1.97, 95% CI 0.92–4.24, $P = 0.082$).

The total duration of breastfeeding affected the risk of sensitization of the infant according to the allergy status of the mother (Fig. 1a). The risk of sensitization in infants with allergic mothers breastfeeding over 6 months increased as compared with those breastfed for $<$ 6 months (OR = 4.83, 95% CI 1.52–15.38, $P = 0.005$). The same was seen in the case of mothers with positive SPT (OR = 3.84, 95% CI 1.00–14.76, $P = 0.041$). Exclusive breastfeeding followed the same pattern (Fig. 1b), and a significant interaction between the allergy status of the mother and the duration of exclusive breastfeeding (interaction test $P = 0.025$) was detected. Infants of allergic mothers had a higher risk of sensitization when exclusively breastfed over 2.5 months as compared with those who were exclusively breastfed for $<$ 2.5 months (OR = 3.43, 95% CI 1.19–9.89, $P = 0.018$).

Effects of probiotics on breast milk composition

We found that TGF-β2 tended to be higher in the colostrum of the mothers in the probiotic group as compared with those on placebo (Table 2). No such trend was detected with regard to other cytokines (Table 2). At 1 month, no differences were seen.

A similar result was obtained in the subgroup of allergic mothers in the probiotic ($n = 21$) and placebo ($n = 22$) groups. Allergic mothers receiving probiotics exhibited higher amounts of TGF-β2 than allergic mothers in the placebo group, 1784.5 vs. 1142.4, respectively (probiotic/placebo ratio = 1.56, $P = 0.094$).

Probiotic effect on sensitization of the infant depends on the allergy risk

There was no difference between infant sensitization in the probiotic vs. the placebo group at the age of 12 months

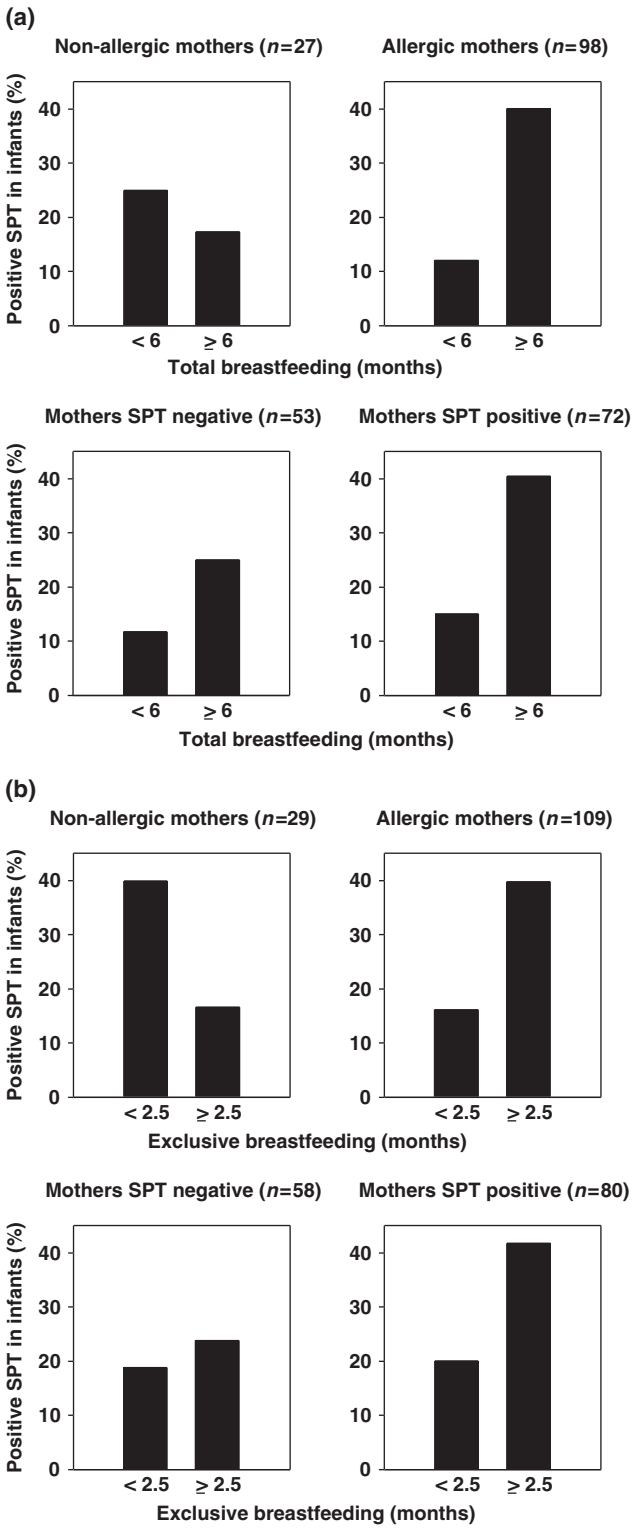

Fig. 1. (a) Positive SPT (%) in infants according to the allergy and SPT status of mother and duration of total breastfeeding. (b) Positive SPT (%) in infants according to the allergy and SPT status of mother and duration of exclusive breastfeeding. SPT, skin prick test.

(29% vs. 31%, respectively, OR = 0.92, 95% CI 0.45–1.90, $P = 0.825$). However, probiotic supplementation had a significant protective effect against sensitization in a subgroup of infants with a high hereditary risk due to maternal sensitization: 26% of the infants in the probiotic group vs. 50% in the placebo group (OR = 0.34, 95% CI 0.13–0.88, $P = 0.023$) tested SPT-positive when the mother was SPT-positive. When the mother had allergic disease, 29.3% of the infants in the probiotic group and 35.8% in the placebo group tested SPT-positive at the age of 12 months (OR = 0.74, 95% CI 0.22–1.65, $P = 0.462$).

Atopic eczema was diagnosed at the age of 12 months in 13.6% of the infants: 9.7% in the probiotic and 17.6% in the placebo group ($P = 0.131$).

Effect of maternal allergic disease, probiotic and breast milk cytokines – evaluation of a connection?

As shown above, duration of breastfeeding in conjunction with an allergic disease and positive SPT or barely positive SPT result in the mother may increase the risk of sensitization of the infant. Again, a low level of TGF-β2 in the colostrum seemed to be associated with a positive SPT result in the infant. In the lowest tertile (T1) of TGF-β2 in colostrum, 38.9% of the infants had positive SPT as compared with 21.1% and 26.3% of T2 and T3, respectively. On the other hand, probiotics increased the level of TGF-β2 (Table 2) in allergic mothers and lowered the incidence of positive SPT of the infants. Therefore, in mothers with infants at heightened risk of sensitization, probiotics increased the level of TGF-β2 and thereby protected the infant from sensitization.

Discussion

In the present study we established that maternal SPT reactivity, accompanied by allergic disease may increase infant vulnerability to sensitization. Interestingly, this development appears to be connected to lactation; receiving breast milk from atopic mothers increases the likelihood of sensitization. In the same vein, protection from sensitization is provided by probiotics in this high-risk group.

The natural course of atopy was formerly conceived of as one entity, where sensitization invariably leads to atopic eczema and finally to asthma [26]. The current view based on recent population studies, however, suggests that sensitization and atopic disease may have different underlying determinants within this cascade [27]. Hence allergy prevention strategies have also been fraught with confusion over measures and targets. To take an example, IgE-mediated sensitization, but not exposure, to cat allergen has been shown to be associated with the development of childhood asthma [28], while another study showed a reduction of allergen exposure by specific

Table 2. Colostrum and 1 month breast milk cytokines (sCD14 as μg/mL, others as pg/mL)

			Ratio Probiotic/Placebo		
	Probiotic group	Placebo group	Mean	95% CI	*P*
sCD14					
Colostrum*	28.80	29.78	0.97	0.72–1.29	0.817
1 month†	5.60	5.60	1.00	0.85–1.18	0.985
TGF-β2					
Colostrum	**1792**	**1194**	**1.50**	**0.96–2.34**	**0.073**
1 month	1093	1080	1.01	0.73–1.40	0.940
IFN-γ					
Colostrum	179.7	132.6	1.36	0.79–2.33	0.266
1 month	211.0	213.0	0.99	0.86–1.14	0.896
TNF-α					
Colostrum	13.65	12.05	1.13	0.74–1.75	0.563
1 month	12.39	11.60	1.07	0.90–1.27	0.446
IL-10					
Colostrum	12.01	10.92	1.10	0.73–1.65	0.641
1 month	14.00	13.37	1.05	0.88–1.24	0.587
IL-6					
Colostrum	75.73	50.49	1.50	0.92–2.45	0.103
1 month	24.60	22.38	1.10	0.86–1.41	0.447
IL-4					
Colostrum	25.81	20.16	1.28	0.78–2.10	0.320
1 month	25.20	22.94	1.10	0.83–1.46	0.515
IL-2					
Colostrum	46.88	38.14	1.23	0.80–1.89	0.341
1 month	32.08	29.71	1.08	0.89–1.31	0.436

*N = 29–32 in probiotic group and N = 24–26 in placebo group.
†N = 35–38 in probiotic group and N = 30 in placebo group.
The results for study groups are given as geometric means and the group comparison as ratio probiotic/placebo with 95% confidence interval (CI).
sCD14, soluble CD14.

avoidance of house dust mite and food allergens to prevent allergic diseases, particularly in sensitized children [29]. Thus, the clinical importance of our finding of a higher SPT reactivity in infants at risk needs to be interpreted with caution.

The same holds true for the allergy protection potential of breastfeeding [3]. In a recent study, breastfeeding for 4 months or more diminished the risk of eczema and postponed the onset of asthma [4], while a study of a New Zealand birth cohort demonstrated, in contrast, that children breastfed for more than 4 weeks were more likely to develop atopic sensitization and asthma as compared with those not breastfed [6]. In a more longitudinal setting, Matheson et al. [30] reported that high-risk infants, exclusively breastfed at least 3 months, showed lower risk of asthma before the age of 7 years, but higher risk of asthma after this as compared with those with a shorter period of exclusive breastfeeding. The results of the present study suggest that the impact of breastfeeding on infant sensitization can be explained by the atopy and allergy status of the mother on the one hand and breast milk composition on the other. Indeed, breast milk varies greatly from mother to mother, and no uniform composition can be identified. Distinctions may be due to nutrients [31], but also to protective factors such as secretory IgA [32] and immunomodulatory molecules such as TGF-β [9]. Allergic mothers have a lower TGF-β2 concentration in their breast milk [9]; TGF-β is a key factor in increasing IgA production on the mucosa, thereby inducing oral tolerance [33, 34]. Earlier data, supported by our present findings, suggest that supplemented probiotics [10, 35] enhance the breast milk composition by creating an anti-inflammatory milieu. Here, probiotic supplementation tended to increase the concentration of TGF-β2 in breast milk.

Probiotics belong among the promising means of combating the allergy epidemic, the underlying concept being that the gut microbiota strongly guide the maturation processes of infant immunity [18]. During this vulnerable period the mucosal immune system is functionally immature, this being manifested as higher intestinal permeability [18]. Furthermore, uptake of intact macromolecules increases due to the elevated binding of antigens to the immature gut wall [18]. Also defencelessness against adherence and translocation of pathogens is high, and the infant may be susceptible to infection and inflammation [18]. Hence the most likely explanation for the protective effects of probiotics may be that specific probiotics are capable of reducing allergic inflammation [36], which was also observed in this study because anti-inflammatory TGF-β2 was increased in breast milk via probiotic administration. In addition, probiotics seem to have effects on antigen degradation, and they are able to enhance the gut barrier and balance aberrancies in the microbiota [37].

In this study probiotic supplementation had no effect on breast milk cytokines other than colostrum TGF-β2, possibly by merit of the probiotics selected. Indeed, different probiotic species have diverse effects, which can also vary between the bacteria in the same strain: cytokine production induced by intestinal bifidobacteria is strain-specific [38], but bifidobacteria strains may induce distinct and even opposite responses [38]. For example, production of IL-10 by intestinal dendritic cells appears to be up-regulated following stimulation with bifidobacteria strains [39] regularly detected in the breastfed infant's gut, while some strains, e.g. *Bifidobacterium adolescentis*, may be pro-inflammatory [38]. Also the age of the host influences responses to microbial stimuli: children seem to be more receptive than adults [16]. Moreover, the presence or absence of intestinal inflammation impacts the adherence properties of probiotics as well as their immunomodulatory potential. Children with atopic eczema, cow's milk allergy [40] and increased

intestinal permeability [41] appear to benefit, while those with non-atopic eczema do not [42].

In the present study we discovered that the infants of allergic mothers, specifically when the SPT was positive, had a higher risk of sensitization at the age of 12 months. However, this risk could be reduced by means of probiotic supplementation during pregnancy and lactation. Thus, the critical period of pregnancy and breastfeeding may be the window of opportunity in allergy prevention. More active measures such as probiotic supplementation can complement the earlier means of passive avoidance. Intervention studies are needed to resolve the discrepancies still prevailing in the field of allergy prevention.

Acknowledgements

The authors would like to thank research nurses Ulla-Maija Eriksson, Sari Laksio and Johanna Hvitfelt-Koskelainen for participating in study visits, Satu Leinonen for technical assistance, Tuija Poussa for statistical consultations and Robert MacGilleon for revision of the English language.

Sources of funding: Sigrid Juselius Foundation, Academy of Finland, The Social Insurance Institution of Finland.

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