

Anna Hogendorf^{1,2}, Anna Stańczyk-Przyłuska^{2,3}, Katarzyna Siniewicz-Luzeńczyk^{2,3},
Magdalena Wiszniewska^{2,3}, Jerzy Arendarczyk^{2,3}, Małgorzata Banasik⁴,
Wojciech Fendler¹, Marek Kowalski⁵, Krzysztof Zeman^{2,3}

IS THERE ANY ASSOCIATION BETWEEN SECRETORY IgA AND LACTOFERRIN CONCENTRATION IN MATURE HUMAN MILK AND FOOD ALLERGY IN BREASTFED CHILDREN?*

CZY ISTNIEJE ZWIĄZEK POMIĘDZY STĘŻENIEM WYDZIELNICZEJ IMMUNOGLOBULINY A I LAKTOFERINY W DOJRZAŁYM MLEKU LUDZKIM A WYSTĄPIENIEM ALERGII POKARMOWEJ U DZIECI KARMIONYCH PIERSIĄ?

¹Department of Pediatrics, Oncology, Hematology and Diabetology,
Medical University of Lodz, Poland

²Department of Pediatrics, Preventive Cardiology and Immunology of Developmental Age,
Medical University of Lodz, Poland

³Polish Mother's Memorial Hospital Research Institute, Lodz, Poland,

⁴APC Laboratory, Lodz, Poland

⁵Department of Immunology, Rheumatology and Allergy,
Medical University of Lodz, Poland

Abstract

Background: Breastfeeding is recommended as a protective method against the development of allergy. However, some studies have reported an increased risk of allergies development in breastfed infants of atopic mothers, which implies that atopic mothers may have an altered composition of breast milk.

Aim: The aim of the study was to determine the concentration of secretory immunoglobulin A (S-IgA) and lactoferrin in human mature milk and to evaluate the association between the levels of these proteins in breast milk with food allergy in children, depending on the allergy status of the breastfeeding mother.

Material and methods: Medical data was collected from birth to 24 months of age from 84 mother-child pairs participating in an EU-funded project "EuroPrevall – The prevalence, cost and basis of food allergy across Europe". The diagnosis of food allergy in children was based on the positive result of a double-blind placebo-controlled food challenge (DBPCFC).

S-IgA and lactoferrin levels were measured in the whey of mature breast milk with commercial enzyme-linked immunosorbent assay (ELISA) kits. Statistical analysis (the U Mann-Whitney and Kruskal-Wallis tests as well as the Spearman's rank correlation coefficient) was performed using STATISTICA 8.0 PL (Statsoft, Tulsa, USA).

Results: Ten out of eighty four participating children had positive skin prick tests (SPT) and/or sIgE to food antigens and in 7 (8.4%) DBPCFC confirmed food allergy. The median concentration of S-IgA was 476.83 µg/ml (range 6.51-1359.61 µg/ml). The median concentration of Lf was 15.68 µg/ml (range 11.68-36.43 µg/ml). The concentrations of S-IgA and Lf showed a moderate, negative, correlation $R=-0.28$; $p=0.05$.

Conclusions: Mature breast milk of mothers of children with food allergy and of healthy children showed similar concentrations of both proteins. The level of S-IgA in the mature milk of mothers with atopic allergy was significantly lower, compared to non-atopic mothers. More studies are needed to reveal the mystery of the lack of protective effect of breastfeeding on allergy development in children.

Key words: breast milk, secretory IgA, lactoferrin, allergy

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Streszczenie

Wstęp: Karmienie piersią jest uważane za jedną z metod prewencji pierwotnej chorób alergicznych. Jednakże wyniki niektórych badań wskazują na większe ryzyko wystąpienia alergii u dzieci karmionych piersią przez matki chorujące na choroby atopowe. To sugeruje, że matki z atopią i matki bez atopii mogą mieć różną zawartość czynników immunologicznych w mleku.

Cel: Celem pracy było określenie stężenia wydzielniczej immunoglobuliny A oraz laktoferyny w dojrzałym mleku ludzkim, a także zbadanie czy istnieją różnice między stężeniem wyżej wymienionych czynników w mleku matek dzieci, u których w ciągu pierwszych 24 miesięcy życia zdiagnozowano alergię pokarmową a stężeniem tych czynników w mleku matek dzieci bez alergii.

Materiał i metody: Badaniem objęte zostały 84 matki karmiące piersią oraz ich dzieci urodzone w latach 2005-2007, wchodzące w skład polskiej kohorty badania „EuroPrevall”. Obserwacja badanej grupy dzieci obejmowała pierwsze 24 miesiące życia dziecka. Stężenie wydzielniczej immunoglobuliny A (S-IgA) oraz laktoferyny (Lf) w mleku oznaczano ilościową metodą immunoenzymatyczną ELISA. Analiza statystyczna (test U Mann’a-Whitney’a, analiza nieparametryczna Kruskala-Wallisa oraz analiza Spearmana) dokonana została przy użyciu programu STATISTICA 8.0 PL (Statsoft, Tulsa, USA).

Wyniki: U dziesięciu spośród 84 dzieci (11,9%) stwierdzono dodatnie punktowe testy skórne (SPT) i/lub sIgE przeciwko alergenom pokarmowym. U 7 spośród 84 dzieci (8,4%) rozpoznano alergię pokarmową na podstawie dodatniego wyniku podwójnie ślepej próby prowokacyjnej z pokarmem kontrolowanej placebo (DBPCFC). Mediana stężenia S-IgA wynosiła 476,83 µg/ml (zakres 6,51-1359,61 µg/ml), a laktoferyny (Lf) 15,68 µg/ml (zakres 11,68-36,43 µg/ml). Stężenia S-IgA i Lf wykazywały umiarkowaną korelację ujemną, $R=-0,28$; $p=0,05$.

Wnioski: Nie stwierdzono, aby mleko matek dzieci, u których w ciągu pierwszych 24 miesięcy życia rozpoznano alergię pokarmową, różniło się pod względem zawartości wydzielniczej IgA i laktoferyny od mleka matek dzieci bez alergii. W porównaniu z matkami bez alergii, matki z alergią atopową miały znacząco niższe stężenie S-IgA w mleku.

Znaczenie karmienia piersią w prewencji rozwoju chorób alergicznych u dzieci wymaga dalszych badań.

Słowa kluczowe: mleko ludzkie, wydzielnicza IgA, laktoferyna, alergologia

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INTRODUCTION

The role of breastfeeding in the primary prevention of allergy is still a subject of a heated debate. The lack of protective effect of human milk against allergies is observed by pediatricians and general physicians in routine practice, but it is also visible in epidemiological studies conducted in developed countries. Some birth cohort studies have even reported an increased risk of the development of atopic disease in children breastfed by atopic mothers (1). As maternal immunity strongly influences the developing gastrointestinal and immune system of the infant, it is likely that this controversy may be explained by individual variability of the immune factors' content in the milk, particularly of those associated with development of oral tolerance.

Breast milk secretory immunoglobulin A (S-IgA) of maternal origin comprises 90% of all human milk immunoglobulin (2). Together with lactoferrin (Lf) it may constitute as much as 30-40% of human milk protein (3). The exclusively breastfed infant ingests from 0,5 to 1 g S-IgA daily (3). Specific human milk S-IgA is believed to reduce the entry of food antigens through mucosal surface of infants' gastrointestinal tract and lower the risk for sensitization in the time when production of infants' S-IgA is minimal (3). It was shown that maternal B cells, activated in the gut-associated lymphoid tissue (GALT)

and bronchus-associated lymphoid tissue (BALT) systems of the mother, circulate within the blood which leads to the production of antigen specific S-IgA in distant mucosa and exocrine glands e.g. mammary gland (4). A significant proportion of breast milk B cells undergo terminal plasma cell differentiation and secrete Ig spontaneously into breast milk (5). Some fractions of S-IgA is also transported and excreted from breast milk by maternal macrophages (6). It is possible due to the existence of the so called "enteromammary" and "bronchomammary" links that the milk contains S-IgA specific antibodies (Abs) against all of the antigens that the mother had contact with through the oral or respiratory route and ensures passive protection of the infant's immature gut barrier (7). Although S-IgA in the human milk has been studied previously, its role in oral tolerance among breastfed children remains unresolved due to conflicting results (8, 9, 10). Human milk lactoferrin (Lf) is an important factor of the innate immunity with immunomodulatory activities. In addition to its antioxidant, bacteriostatic and bactericidal properties Lf may act as a regulator of immune and inflammatory responses and may protect against Th-mediated diseases through correction of Th1/Th2 cytokine imbalance (11). The role of Lf in human breast milk has not been previously studied in relation to the development of allergy in breastfed children.

AIM

The aim of the study was to determine the concentration of secretory immunoglobulin A (S-IgA) and lactoferrin in human mature milk and to evaluate the association between the levels of these proteins in breast milk with food allergy in children depending on the allergy status of the breastfeeding mother.

MATERIAL AND METHODS

Data on breastfeeding, allergic diseases of the mother and symptoms of food allergy in their children were collected prospectively from birth to 24 months of age from 84 mothers participating in the Polish birth cohort of an EU-funded multidisciplinary integrated project "EuroPrevall – The prevalence, cost and basis of food allergy across Europe, Work Package 1.1- Pediatric Food Allergy- characteristics of children developing food allergy", using standardized baseline and follow-up questionnaires. The clinical assessment, as well as double blind placebo controlled food challenges were performed in the Polish study centre of 1.1 work package of EuroPrevall study- the Department of Pediatrics, Preventive Cardiology and Immunology of Developmental Age, Medical University of Lodz, Poland.

In children suspected of food allergy, skin prick tests (SPT) were performed followed by the measurement of specific IgE (sIgE) to food allergens using the Phadia Immuno CAP 250 system (Phadia Diagnostics, Uppsala, Sweden). All measurements were sent out to be performed centrally in the Department of Paediatric Pneumology and Immunology, Charite University Medical Center, Berlin, Germany. The diagnosis of food allergy in children was based on a double-blind placebo controlled food challenge (DBPCFC) according to EuroPrevall's study protocol (12).

Mothers reporting clinical symptoms of allergy were referred to as "allergic" and mothers with a history of allergy confirmed by positive SPT or sIgE were considered to as "atopic" (based on history and medical records).

The study was approved by the Ethical Committee of the Medical University of Lodz and all parents of the children participating had expressed their informed consent by signing relevant forms.

Breast milk sampling

Breast milk samples of mature milk were collected during different periods of lactation.

The milk was collected only once from each mother, during her visit in the clinic related to the probable onset of allergy symptoms in the child, a routine check-up or a questionnaire visit scheduled according to the EuroPrevall protocol.

The mothers were asked to express their milk manually or using manual breast milk pump (Avent ISIS 12080, Suffolk, UK). The milk (6 ml) was collected into 3 sterile polypropylene tubes and stored at -20°C and -80°C, until analysis.

Determination of S-IgA and lactoferrin levels in milk

After thawing, the samples were centrifuged at 680 g for 10 minutes at 4°C, after which the fatty layer and cellular

elements were discarded. The whey was then centrifuged at 10 000 g for 30 minutes at 4°C and supernatants were used for analysis. Quantitative measurement of S-IgA and Lf levels was performed using commercial enzyme-linked immunosorbent assay (ELISA) kits: Secretory IgA ImmunoChrom ELISA Kit ImmunoChrom GmbH Cat No. IC6100 for the determination of S-IgA in stool and saliva and assay for human lactoferrin BIOXYTECH® LactofEIA™, OxisResearch®, Cat No. 21015.

Statistical analysis

Nominal variables are presented as percentages and continuous variables are given as medians and interquartile ranges (IQRs) due to non-normal distribution (verified by means of W. Shapiro-Wilks test). The U Mann-Whitney test or Kruskal-Wallis nonparametric analysis of variance were used for pairwise and multiple group comparisons respectively. Spearman's rank correlation coefficient was used for correlation analysis. The threshold of p value considered as significant was set at <0.05. Statistical analysis was performed using STATISTICA 8.0 PL (Statsoft, Tulsa, USA).

RESULTS

Sample characteristics

Among 84 participating children 42 were boys (50%). One third (N=26; 30.95%) of the children were born by cesarean section. All but four (N=80; 95.24%) were born full-term. The average length of pregnancy was 38.4±1.53 weeks. The average birth weight was 3354.6±426.6 g. The average length of the breastfeeding period was 15.36±6.37 months. The median age of children at milk collection was 7.33 (IQR 3.6-13.1) months. The median children's age at milk collection did not significantly differ between children of allergic and non-allergic mothers [8.1 months (IQR 2.5-13.77) vs 7.27 months (IQR 3.6-12.5), p=0.78]. No difference was also seen between children without any symptoms of allergy, children with symptoms and positive SPT and/or sIgE and children with positive DBPCFC [7.85 months (IQR 3.60-13.73) vs 5.45 months (IQR 2.77-7.53) vs 6.83 months (IQR 4.73-8.10), p=0.23].

Over 1/4 mothers (N=25; 28%) reported symptoms of allergy. The majority of them (N=16; 69.5%) reported symptoms of allergic rhinitis and/or conjunctivitis, 7 (30.4%) had a history of adverse reaction to food, 4 (17.3%) reported hypersensitivity to medications (2 to non-steroid anti-inflammatory drugs (NSAID), 1 to both NSAID and penicillin antibiotics, 2 (8.6%) reported the appearance of atopic eczema, 1 was allergic to propolis), in 4 (17.3%) a systemic reaction occurred after the bee sting, 3 (13%) observed local reaction after application of cosmetic creams. In 9 cases sensitization was confirmed on the basis of a positive results of SPT and/or sIgE.

Ten out of eighty four (11.9%) participating children had positive SPT and/or sIgE to food antigens. In 7 out of 10 such children DBPCFC confirmed the diagnosis of food allergy.

Among 7 children with positive DBPCFC (5 girls and 2 boys), there were 2 cases of cow's milk allergy, 2 of hen's egg allergy, 2 cases of both cow's milk and hens' egg and

one case of allergy to 3 allergens: cow's milk and hens' egg and peanuts. Among children with positive DBPCFC 5 were born to allergic mothers (71.4%), but only in 2 of the mothers atopy was confirmed by a positive SPT result with inhaled allergens.

IgA and lactoferrin were detectable in 98% of milk samples. Median concentration of S-IgA was 476.83 µg/ml (range 6.51-1359.61 µg/ml). Median concentration of Lf was 15.68 µg/ml (range 11.68-36.43 µg/ml). The concentrations of S-IgA and Lf showed a moderate, negative, correlation $R=-0.28$; $p=0.05$.

The level of S-IgA in the mature milk of mothers with confirmed atopy was significantly lower (median concentration 476.836 µg/ml (IQR 209.2-678.53 µg/ml)),

than in non-atopic mothers (782.47 µg/ml (IQR 614-916.69 µg/ml); $p=0.02$; table I, Fig. 1). There was a higher concentration of lactoferrin in milk of atopic compared to non-atopic women [17.790 µg/ml (IQR 15.657-24.33 µg/ml) vs 22.0 µg/ml (IQR 18.13-30.66 µg/ml)], but the difference was not statistically significant ($p=0.1$; Fig. 2).

There were no significant differences in the concentration of S-IgA and lactoferrin in the mature breast milk of mothers of children with symptoms of allergy and positive SPT and/or sIgE (table II). No differences were also observed between the concentration of S-IgA and lactoferrin in the mature breast milk of mothers of children with positive DBPCFC and in milk of mothers of healthy children.

Table I. Secretory IgA and lactoferrin concentration in mature milk of atopic and non-atopic mothers (µg/ml).

Tabela I. Stężenie wydzielniczej IgA i laktoferyny w mleku matek z alergią atopową i matek bez alergii atopowej.

	Non-atopic mothers <i>Matki bez atopii</i> (n=61)	Atopic mothers <i>Matki z atopią</i> (n=9)	p
S-IgA	782.47 (614.04-916.69)	476.83 (209.20-678.53)	0.02
Lactoferrin	17.79 (15.65-24.33)	22.00 (18.13-30.66)	0.1

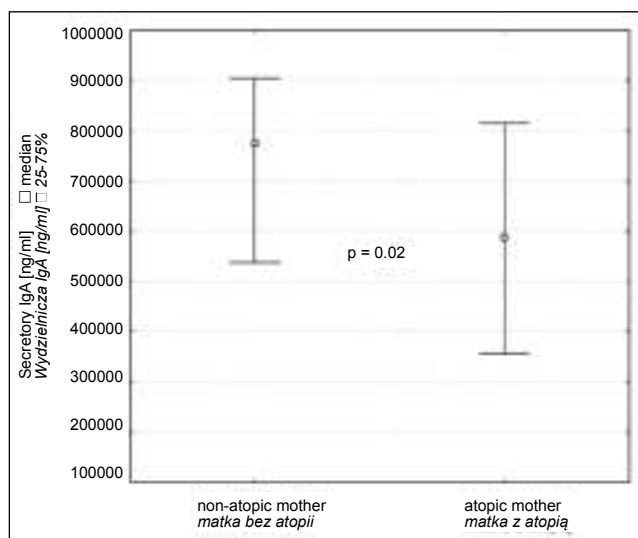


Fig. 1. Comparison of total secretory IgA concentration (ng/ml) in mature milk of atopic versus non-atopic mothers.

Ryc. 1. Porównanie stężenia wydzielniczej IgA (ng/ml) w mleku dojrzałym matek z alergią atopową i matek bez alergii atopowej.

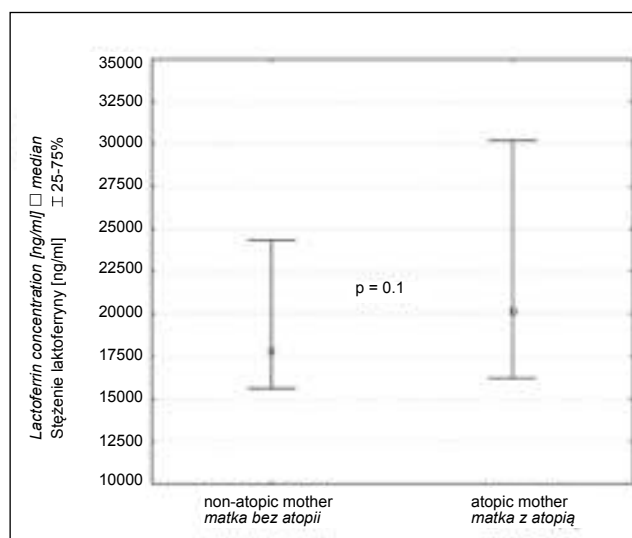


Fig. 2. Comparison of lactoferrin concentration in mature milk of atopic and non-atopic mothers.

Ryc. 2. Porównanie stężenia laktoferyny (ng/ml) w mleku dojrzałym matek z alergią atopową i matek bez alergii atopowej.

Table II. Comparison of secretory IgA (S-IgA) and lactoferrin concentration in mature milk of mothers of children with positive DBPCFC, positive SPT and/or sIgE and children with no symptoms of allergy.

Tabela II. Porównanie stężenia wydzielniczej IgA (S-IgA) i laktoferyny w mleku dojrzałym matek dzieci z dodatnim wynikiem DBPCFC, SPT i/lub sIgE i dzieci bez objawów alergii.

	S-IgA (µg/ml)	Lactoferrin (µg/ml)
Positive DBPCFC (n=7)	529.11 (290.81-891.80)	22.0 (17.48-32.52)
Positive SPT/sIgE (n=10)	841.40 (623.73-1084.19)	21.0 (13.9-24.36)
Control group (n=74)	713.88 (481.76-874.71)	17.79 (15.65-25.71)

DISCUSSION

As the intestinal barrier functions are immature in early infancy, an increased load of foreign proteins taken up through the oral route can gain easier access to the systemic circulation, which may lead to hypersensitivity. Lower amounts of antigens in breast milk, processed by the mother's intestinal mucosa and presented in conjunction with specific antibodies, may induce maturation and conditioning of infants' immune system as well as promoting tolerance to food-born antigens (2). Thus, S-IgA acts both as a neutralizing agent (by means of a process called "immune exclusion") and as an immunomodulator.

The results of this study indicate that atopic mothers had lower concentration of total S-IgA in their mature milk. Some previous studies revealed that high levels of human milk S-IgA are protective against the development of cow's milk allergy and that infants with food allergy receive smaller quantities of specific or total IgA antibodies in colostrums, (8, 9, 13). Specific antibodies in colostrums however, had not been shown to correlate with the atopic status of the mother (8). Other authors failed to prove the different content of total IgA in milk of allergy versus non-allergic mothers (14).

In our study the lower concentration of total S-IgA in mature milk had no influence on the development of allergy in the first 24 months of life. A similar finding was reported by Böttcher et al. S-IgA levels as well as studied cytokines and chemokines did not affect the development of SPT-confirmed sensitization during the first 2 years of life (10). The lack of association between S-IgA concentration in breast milk and positive SPT or symptoms of allergy was also reported by Duchon et al. (15).

It is also possible that higher concentration of S-IgA in colostrum showed a stronger effect on oral tolerance development than S-IgA of mature milk.

As shown by the above, the discrepancy between our results and the results of other studies may be also due to some limitations of the study (e.g. due to small number of atopic mothers) and different methodological approach. Unlike other studies, we used the double blind placebo controlled food challenge (DBPCFC) – a gold standard method to confirm the food allergy diagnosis. This resulted in a smaller number of children with true food allergy. What is more, this study demonstrated the lack of a preventive effect of breastfeeding in children breastfed for a strikingly long period of time (mean length of breastfeeding was 15.36 months). Regardless of prolonged breastfeeding in 10 (11.9%) of the children, symptoms of IgE mediated reaction were present.

The additional aim of this study was to determine a possible protective effect of other important breast milk protein of immunoregulatory potential – human lactoferrin. This is to our knowledge the first study including human lactoferrin in the debate on the influence of breast milk factors and allergy development in children in such an approach. Synthesized by epithelial cells and granulocytes, Lf is present in many mucosal secretions and has multiple functions, such as regulation of iron absorption by intestinal epithelium, epithelial cell

proliferation and immune defense mechanisms (16, 17). In breast milk, where it was first discovered, it is found at the highest levels of about 7 g/l and at a lower level in mature milk (between 2 and 4 g/l) (18). The existence of highly specific receptor on the brush border membrane of human infant's intestine may facilitate cellular uptake of breast milk Lf and iron absorption (19), inducing cell proliferation, particularly within the mucosa and GALT (20). Lf shows immunomodulatory properties by influencing proliferation, maturation and differentiation of T cells towards Th1 or Th2 phenotype (11) and affecting maturation of B cell and their transformation into antigen presenting cells (21). Lf increases the number of CD4+ cells in the lymphatic tissue and the *lamina propria* of murine intestine (22). Additionally, through its direct iron-binding capacity, it inhibits intestinal growth of pathogenic iron-dependent bacterial strains such as *Escherichia*, *Clostridium*, *Bacterioides*, *Staphylococcus*, *Salmonella* (23) promoting the development of probiotic flora (24).

Despite the fact that Lf may have the immunoregulatory potential in allergic disorders as shown *in vitro*, its role in the development of allergic diseases in breastfed children has not been confirmed in our study, although a trial with greater statistical power would be required to completely rule out this association. Our study also showed that atopic mothers did not have significantly higher concentration of Lf in breast milk, although atopic mothers did show slightly higher concentrations of this protein ($p=0.10$).

It must be emphasized that in the presented study, only mature milk was analyzed. It is a well-established fact that the composition of human milk varies during the course of lactation. As previous studies show, the concentration of immune factors differs profoundly between individual women, even if the milk is collected strictly at the same time points, which makes a cross-sectional analysis difficult (25-27). Allergy, infections, stress and supplementation with probiotics are suggested to be the leading cause of this phenomenon (28). The milk was collected only once from each mother, during check-up or a questionnaire visit scheduled according to EuroPrevall study protocol. The analyzed mature milk shows a rather "stable" content, especially over 30 day of lactation (29). Additionally, the lack of correlation between the time of collection of samples and the concentration of S-IgA and Lf increases the credibility of the analysis.

CONCLUSIONS

Mature breast milk of mothers of children with food allergy and healthy children showed similar concentrations of both proteins. The level of S-IgA in the mature milk of mothers with atopic allergy was significantly lower, comparing to non-atopic mothers. More studies are needed to reveal the mystery of lack of protective effect of breastfeeding on allergy development in children.

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Authors' contributions/Wkład Autorów

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Address for correspondence:

Anna Hogendorf

Department of Pediatrics, Oncology,

Hematology and Diabetology

Medical University of Lodz

Sporna Str. 36/50, Łódź, Poland

tel. (48 42) 617-77-50, fax (48 42) 617-79-89

e-mail: anna.hogendorf@gmail.com