Association of the Protein Z ATG haplotype with symptomatic non-vascular stroke or thromboembolism in white children – a family-based cohort study

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Running title: pediatric stroke/TE and protein Z haplotypes

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Summary

To clarify the role of protein Z (PZ) in children with stroke/thromboembolism (TE) the present haplotype (HT)-based family study was performed. We genotyped 365 pediatric stroke/TE families (stroke n=216; TE n=149) for four single nucleotide polymorphisms (SNPs: rs3024718, rs3024731, rs3024772 and rs3024778) to assess the association between genetic variation within a conserved block of linkage disequilibrium harboring the PZ gene and pediatric TE. Association was assessed using the Transmission Disequilibrium Test (TDT), corrected for multiple testing (permutation testing: HAPLOVIEW). In addition, PZ antigen was determined and correlated with carriership of PZ haplotypes and the FV G1691A mutation. Rs3024718, rs3024731, rs3024772 are in tight linkage disequilibrium (LD) and define four haplotypes, capturing 97% of the genetic variation for this LD block. HT1 (ATG) was significantly over-transmitted from parents to affected offspring (HT frequency 73.5%, T:U 122:80, $\chi^2=8.791$, $p=0.003$). The ATG risk haplotype was significantly correlated with higher PZ antigen levels. Multivariate analysis adjusted for age, sex, established thrombophilias, smoking, fibrinogen and PZ levels revealed a significant association of the ATG haplotype and TE in children (OR/95%CI: 1.4/1.08-1.93). Our results suggest that the ATG haplotype of the PZ gene is a genetic marker for symptomatic TE in white German children.

Keywords: protein Z haplotypes, protein Z antigen levels, pediatric stroke-thromboembolism, family study
Introduction

Stroke in children is a heterogeneous disorder associated with significant morbidity and mortality. The incidence has been estimated at 1 per 4000 neonates and has ranged from 1 per 7000 to 1 per 70,000 for older children. The most frequent reported risk factors for stroke in neonates, children and adolescents include underlying medical conditions such as cardiac disorders, metabolic diseases, cerebro-vascular pathologies, and infections diseases. In addition within the last decade, many genetic and acquired prothrombotic abnormalities have been evaluated in children with cerebrovascular disease and have been found more common in children with stroke compared with healthy children.

The gene for human protein Z (PZ) is localized to chromosome 13q34 where the genes for factors VII and X exist side by side, it spans approximately 14 kb consisting of 9 exons, including one alternative exon. PZ is a single-chain glycoprotein of 62 kDa and is synthesized in the liver. It was first purified from bovine plasma and later found also in humans. PZ is a vitamin K-dependent protein such as factors II, VII, IX and X and the inhibitors protein C and S. The complete amino acid sequence has been described by Ichinose et al. in 1990 and Sejima et al., and the age-dependent plasma levels in healthy individuals ranged from 1.4 to 2.9 μg/ml. Initially the physiological function of protein Z was unclear: In-vitro studies showed that bovine PZ could promote the assembly of thrombin with phospholipid surfaces, thus enhancing coagulation. In contrast, the human form of PZ binds thrombin poorly with very little impact on the association of thrombin binding with phospholipids. More recent studies have shown that PZ forms a calcium-ion dependent complex with factor Xa on the phospholipid surface and thereby serves as a cofactor for the inhibition of factor Xa by a protein Z-dependent protease inhibitor (ZPI).

Protein Z phenotypes are considered to be influenced by genes, by acute phase reactions, and environmental factors. Thus, in a continuation of our previous finding that the phenotypic variation of PZ is significantly attributable to heritability and shared environmental effects in pediatric stroke families, the present haplotype (HT)-based family study was performed to identify additive genetic effects influencing the variability of the PZ phenotype.
Material and Methods

Patients and Methods

In the German study center Muenster, plasma and DNA samples of 365 families from children with non-vascular stroke (n=216) or symptomatic thromboembolism (TE: n=149), i.e. samples of the propositus, non-affected brothers or sisters and biological parents have been collected between January 2000 and December 2007 (Fig. 1). The present study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was approved by the medical ethics committee of the University of Muenster, Germany. With written parental consent, term neonates and children with confirmed diagnosis of TE not older than 18 years at onset, biological brothers and sisters and available parents were enrolled. Stroke subtypes of the children enrolled were reclassified according to explicit predefined criteria based on the TOAST criteria modified for children by substituting vasculopathy for large vessel atherosclerosis.16-18 Premature birth (< 36 gestational weeks), patients older than 18 years at onset, and children with a first stroke of vascular origin, e.g. moyamoya disease, vasculitis, dissection, fibromuscular dysplasia, and focal stenosing arteriopathy, were excluded. In addition, children with missing parents, families with genetically implausible paternity, children lost to follow-up and patients without parental consent were not ascertained. Further exclusion criteria were ongoing liver, renal, or inflammatory diseases, malignancies, and concurrent treatment regimens known to influence PZ levels. In- and exclusion criteria are shown in Fig 1.

In addition, the role of elevated PZ antigen levels in patients and healthy unrelated pediatric controls are evaluated (supplemental material).

Genetic analysis

We identified 4 haplotype tagging SNPs (htSNPs) with a minor allele frequency of > 1% capturing 97.5% of the genetic variation in PZ genes,19 using the genotype information from
the “Centre d'Etude du Polymorphisme Humain (CEPH)” families available from HAPMAP (www.hapmap.org) and the SNPtagger-tool as implemented in HAPLOVIEW. These four hSNPs located in PZ are rs3024718, rs3024731, rs3024772 and rs3024778. Rs3024718 and rs3024772 are in accordance with the polymorphism recently reported by Staton et al., and by Kemkes-Mattes et al. Genotyping was performed using the TaqMan allelic discrimination method on a 384-well HT7900 (Applied Biosystems, Foster City, USA) using 2ng of genomic DNA. For quality control each plate contained 8 positive CEPH-controls and 8 empty wells (NTCs) to ensure genotyping accuracy. Genotyping efficiency was >99.5%. DNA extraction was performed by a spin column procedure (Qiagen) as previously described.

**Blood sample collection**

For the genotype-phenotype correlation study blood sample collection from patients and relatives was done in the morning after a 12-hour fasting period (infants 4-6 hours); samples were drawn by peripheral venipuncture into plastic tubes containing 1/10 by volume of 3.8% trisodium citrate (Sarstedt) and were immediately placed on melting ice. The blood samples from patients were collected 6-12 months after the acute thrombotic event, and at least 6 weeks apart from anticoagulation. Platelet-poor plasma was prepared by centrifugation at 3000 g and 4° C for 2x20 minutes, aliquoted in polystyrene tubes, stored at -70° C and thawed immediately before assay. For the present PZ phenotype study blood samples were drawn from otherwise healthy relatives with normal hemograms and no evidence of further diseases.

**Laboratory analysis**

Total protein Z concentrations were measured along with fibrinogen and D-Dimer levels with ELISA technique (Asserachrom protein Z, Stago) six to twelve months after the acute TE onset and after withdrawal of oral anticoagulation. Pediatric reference values previously reported were used for comparison. As an acute phase reactant, fibrinogen
was measured according to Clauss (Dade Behring BCS analyser). Quantitative D-dimer levels were measured by a latex-enhanced turbimetric test (D-dimer plus, Dade-Behring) using the Dade Behring BCS analyser. Further evaluation included testing for the FII G20210A variant, the FV G1691A mutation, antiphospholipid antibodies (including the lupus anticoagulant at minimum), levels of antithrombin, protein C, and free protein S-antigen, and lipoprotein (a) in all cases.

**Statistical analysis**

Hardy-Weinberg-Equilibrium for each htSNP was tested using $\chi^2$-analysis across all samples. Family-based association was determined using the transmission disequilibrium test (TDT) in 365 trios comprising unaffected parents and the affected child; $^{27}$ haplotypes were inferred using the Expectation Maximization Algorithm (EM-algorithm) as implemented in Haploview (Vs. 4.0). $^{20}$ Significance of association was assessed using a Pearson $\chi^2$-test and corrected for multiple testing using 10,000 permutations as implemented in HAPLOVIEW. Association was assessed using the Transmission Disequilibrium Test (TDT) and corrected for multiple testing using permutation testing.

For correlation analyses between risk haplotype and total protein Z levels in the affected subjects, the individual haplotypes were reconstructed based on phase, since genotype information was available for both parents and the affected child. Distribution of total protein Z was assessed using the Kolmogorov-Smirnoff-Test for normalcy to ensure a normal distribution as a prerequisite for parametric analyses [student’s T-test: values are shown as mean and standard deviation (SD)]. Correlation analyses was performed using one-way analysis of variance (ANOVA), followed by the appropriate post-hoc test (student’s T-test). Furthermore, associations between risk haplotypes and established risk factors for pediatric TE [FII G120210A variant, FV G1691A mutation, antiphospholipid antibodies, antithrombin, protein C, free protein S-antigen, elevated lipoprotein (a)] were compared by $\chi^2$-analysis or by Fisher’s exact test, if appropriate. Multivariate analysis (logistic regression) adjusted for
possible confounders, i.e. age, sex, smoking (yes/no), established inherited thrombophilias including antiphospholipid antibodies, fibrinogen and protein Z antigen levels was performed to investigate the association between PZ risk haplotypes and non-vascular stroke/TE compared with healthy relatives: In this multivariate model, variables known a priori to be important covariates based on previous work, and variables which have shown a p-value of < 0.2 in univariate analysis were incorporated into the final statistical model. Results were expressed as odds ratios (OR) and 95% confidence intervals (CI).

Results

Distribution of haplotype tagging SNPs in the cohorts investigated

The distribution of the four htSNPs is shown in table 1: no statistical significant differences were found between non-vascular stroke trios and trios with thromboembolism with respect to observed/predicted heterozygosity, non-missing genotypes and minor allele frequencies; thus, to increase statistical power both pediatric cohorts were analyzed together. Data of the non-vascular stroke and TE cohorts were additionally shown.

Single point association between PZ htSNPs and non-vascular stroke/TE

Single-point TDT in 365 non-vascular stroke/TE trios did not identify significant associations between rs3024718, rs3024731, rs3024772, or rs3024778 and pediatric non-vascular stroke/TE respectively (for further details see table 2).

Association between PZ haplotypes and non-vascular stroke/TE

In the pooled cohort genotyping of 365 family trios for TE showed that the four selected htSNPs identified the two most common haplotypes in PZ. Rs3024718, rs3024731, rs3024772 are in tight linkage disequilibrium (LD) and define four haplotypes, capturing 97% of the genetic variation for this single LD block capturing the entire PZ gene (Fig. 2a and 2b). Whereas single-point TDT identified no significantly associated SNPs with pediatric non-vascular stroke/TE, haplotype-based association analysis revealed that haplotype HT1 of
the PZ gene (ATG) was significantly over-transmitted from parents to affected children (HT1, ratio of transmissions to non-transmissions of the over-transmitted allele (T:U=122:80, \( \chi^2 = 8.79, p = 0.003 \)). The same was true in the cohort of 216 trios with non-vascular stroke (T:U=73.2:42.2, \( \chi^2 = 8.35, p = 0.004 \)) and a trend in 149 trios with symptomatic TE (T:U=49.5:32.2, \( \chi^2 = 3.66, p = 0.055 \)).

In contrast, in the entire cohort, haplotype HT2 (GAG) was significantly under-transmitted to affected offsprings (HT2, T:U=64.5:94.5, \( \chi^2 = 5.66, p = 0.017 \): for detailed information see Table 3). The association for HT1 in PZ remained significant following permutation testing using 10,000 permutations (HT1, \( \chi^2 = 8.35, p = 0.018 \)), while significance was lost for HT2 (HT2, \( \chi^2 = 3.05, p = 0.253 \)) indicating that the observed under-transmission is likely a statistical fluctuation rather than a true association.

**Correlation between ATG haplotype and PZ concentrations:**

For this analysis, complete data of 1116 individuals derived from non-vascular stroke/TE families (index patient and healthy relatives) were available, with blood samples drawn clearly beyond the acute TE onset. In subjects with the PZ ATG risk haplotype (n=632) PZ levels (mean + SD) were significantly higher compared with those without this haplotype (n=484: 1.64 ± 0.69 \( \mu g/ml \) versus 1.27 ± 0.60 \( \mu g/ml \); \( p < 0.001 \)). In contrast, mean +SD fibrinogen [274+72.3 vs. 273+76.4 mg/dl; \( p = 0.61 \)] and D-dimer levels [0.15+0.22 vs. 0.18+0.45 mg/l; \( p = 0.41 \)] were no different between subjects of the ATG haplotype and those without. The role of elevated PZ antigen levels > 90\(^{th}\) age-dependent percentiles (suppl. Table I) is shown in suppl. Table II: Elevated PZ antigen levels > 90\(^{th}\) age-dependent percentiles in children with non-vascular stroke or venous thromboembolism were significantly more common in patients compared to healthy controls.

No statistical significant difference, however, was found for PZ antigen concentrations in index patients compared with healthy relatives (\( p = 0.56 \)). Heterozygosity of FV G1691A was equally distributed in patients/relatives carrying the ATG haplotype compared to those
without, and PZ levels were no statistically different between FV G1691A heterozygotes versus wild-type carriers [1.46 + 0.64 μg/ml versus 1.49 + 0.69 μg/ml; p=0.675].

Interaction of PZ ATG haplotype with established thrombophilia and further possible co-factors (data for combined analyses, non-vascular stroke and TE patients are separately shown):

In univariate analysis the PZ risk haplotype (ATG) did not show any statistically significant positive association with the overall rate of established thrombophilic risk factors (FII G120210A variant, FV G1691A mutation, antiphospholipid antibodies, antithrombin, protein C, free protein S-antigen, lipoprotein (a): combined data [p=0.29], non-vascular stroke [p=0.38], TE [p=0.11]): factor II G20210A variant (combined data [p=0.71]; non-vascular stroke [p=0.98]; TE [p=0.53]), factor V G1691A mutation (combined data [p=0.18], non-vascular stroke [p=0.09]; TE [p=0.07]), and interestingly, elevated lipoprotein (a) was found less commonly in subjects carrying the risk haplotype compared to those without (combined data [p=0.038]; non-vascular stroke [p=0.031]; TE [p=0.07]). In subsequent multivariate analysis (logistic regression) adjusted for age, sex, smoking, presence of established thrombophilias, protein Z and fibrinogen levels the HT1 haplotype (ATG) was significantly more common in non-vascular stroke/TE patients compared with healthy relatives (combined data [OR/95%CI: 1.4/1.08-1.93]; non-vascular stroke [OR/95%CI: 1.46/1.02-2.1]; TE [OR/95%CI: 1.54/1.01-2.35]).

Discussion

The definition of haplotype blocks of SNPs has been proposed as markers in association studies to efficiently describe human genetic variation. In addition, it has been proposed to distinguish between block haplotypes by a minimal set of HT-tagging SNPs, which would then efficiently describe the variation in the human genome by allowing for genotyping of only a subset of SNP loci. In the present candidate gene association study, whose cohorts comprised white German pediatric non-vascular stroke or TE patients and their biological
parents (nuclear families) we have used the latter approach. In the present cohorts we found that the PZ ATG haplotype is a genetic marker for non-vascular stroke/TE in children, and further that the PZ or a neighboring gene is a susceptibility gene for pediatric non-vascular stroke/TE. In addition, in the present study the ATG risk haplotype was significantly correlated with higher PZ antigen levels compared to subjects not carrying the risk haplotype.

At a first step, we have performed single-point TDT followed by HT-based association analysis: Our observation that single-point TDT did not identify significantly associated SNPs with pediatric non-vascular stroke/TE is in line with observations of Cesari et al., and Sofi et al. that the intron F G79A polymorphism (rs3024718 SNP) is not associated with acute coronary syndrome or ischemic stroke in adults. In a second step, we have performed a HT-based association study, demonstrating that this HT-based analysis revealed the PZ ATG haplotype is associated with pediatric non-vascular stroke/TE. In a third step, we have further investigated this association with pediatric non-vascular stroke/TE in multivariate analysis adjusted for possible confounders such as age, sex, smoking habits, inherited thrombophilias and fibrinogen levels.

A smaller but identical HT subset has been observed in the TE cohort (AT) or non-vascular stroke (ATG) alone, which is likely a consequence of the inaccuracy of the EM-algorithm in smaller study samples. However, the T-allele of rs3024731 uniquely tags HT1 (Figure 2) and is be sufficient to correctly call this haplotype. Along with the observed identical distribution of observed/predicted heterozygosity, percentage of non-missing genotype, and the missing statistical difference between minor allele frequencies, we were confident that haplotypes were similarly distributed between the two pediatric cohorts, and could thus be pooled. In addition to our finding that the ATG HT is a risk marker for non-vascular stroke/TE in white German children, we found that the GAG haplotype was under-transmitted, which either might constitute an independent protective HT or, more likely, a statistical fluctuation mirroring the over-transmission of HT1.

Contradictory data have been reported for protein Z plasma levels associated with coagulation disorders: on the one hand, many disease states have been reported in relation
to PZ deficiency, i.e. ischemic stroke,\textsuperscript{33} unexplained early fetal loss,\textsuperscript{34} and deep venous thrombosis related to factor V Leiden,\textsuperscript{35} but on the other hand, ischemic stroke has also been discussed with normal or elevated PZ levels in adults and children.\textsuperscript{21,26,36,37} Although in the present study higher PZ levels (determined in plasma samples clearly collected beyond the acute disease onset concomitant with normal fibrinogen and D-Dimer levels) are correlated with the PZ ATG risk haplotype, these values are within the pediatric reference ranges previously reported.\textsuperscript{26} Thus, from the family-based association study presented here we only can conclude that the PZ ATG haplotype is not associated with PZ deficiency. In the case-control study performed (suppl. material), however, we were able to demonstrate that elevated PZ antigen levels > 90\textsuperscript{th} age-dependent percentiles are more often found in patients with non-vascular stroke or venous thromboembolism compared with normal controls. Contradictory associations between plasma PZ plasma levels and deep venous thrombosis have also been reported in adults. Whereas Vasse et al. did not observe an association between low protein Z levels and deep venous thrombosis,\textsuperscript{33} Kemkes-Matthes et al. could clearly demonstrate an aggravation of deep venous thrombosis, i.e. an earlier manifestation with more severe thromboembolic complications in adults with PZ-deficiency and FV Leiden carrier status.\textsuperscript{35} In our analysis, however, a statistically significant correlation between lower PZ antigen levels and FV Leiden carrier status could not be shown. Apart from our findings that the ATG haplotype itself is associated with the outcome of interest, e.g. pediatric non-vascular stroke or TE, we speculate that the observed higher protein Z antigen levels in our study might be due to an interaction with the protein Z-dependent protease inhibitor (ZPI). Here we suggest the hypothesis that in children with persistently elevated PZ antigen levels PZ is not completely bound to ZPI, either due to acquired or inherited ZPI deficiency.\textsuperscript{38} ZPI is a hemostatic serpin with anticoagulant activity, which in its deficient state, comparable to antithrombin deficiency, could have thromboembolic consequences.\textsuperscript{7} Recently genetic variations in the ZPI gene have been described\textsuperscript{39,40} and in the present cohorts only preliminary data with respect to ZPI gene analyses were available: four out of 216 patients with non-vascular stroke [1.9\%], three out of 149 children with TE [2.0\%], and nine of 751
unaffected relatives were heterozygous for the silent ZPI gene mutation in exon 3 (1276C>T: rs2232707), and interestingly, in nine of these cases this ZPI gene mutation was combined with the PZ ATG risk haplotype (personal communication). The ZPI 728 C>T mutation and other polymorphisms have not been investigated by us so far.\textsuperscript{40}

Limitations of the present family-based cohort study include i) the non-availability of protein Z values in the entire study cohort and ii) the fact that children in this study were principally white. Thus, at the present stage the association study between protein Z haplotypes and phenotypes is preliminary and should be confirmed by future cohort studies. In addition, since populations differ in prevalence of many complex genetic diseases, such as cardiovascular disease, stroke, and deep venous thrombosis, the data presented here cannot be extrapolated to pediatric non-vascular stroke/TE patients of other ethnicities.

In conclusion, our results suggest that the ATG haplotype of the PZ gene is a genetic marker for symptomatic non-vascular stroke/thromboembolism in white children, and further that the PZ or a neighboring gene is a susceptibility gene for TE in the German family-based cohort investigated. Our observation that the HT-based study design is superior to the single-point approach underlines the necessity to overcome the limitations of single-point association studies in diseases that do not underlie a monogenetic Mendelian inheritance. Finally, our study highlights the complex nature of pediatric non-vascular stroke/TE and the importance of evaluating gene-gene interactions in association studies of complex, polygenic diseases. Further studies in the present cohort are ongoing to investigate the associations between PZ haplotypes and protein Z phenotypes.
Acknowledgement

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Authors contributions and conflict of interest statement

Along with the principal study investigators, e.g. Monika Stoll and Ulrike Nowak-Göttl who act as the guarantors, all other investigators had full access to the data (Birgit Fröhlich, Sabine Thedieck, Andreas Huge) and took part in the design, execution and data analysis, and in writing the report. Monika Stoll and Ulrike Nowak-Göttl were responsible for the statistical analyses. None of the authors listed have financial or personal relationships with other people or organizations that inappropriately influence their actions.
References


Legends:

Figure 1

In figure 1 in- and exclusion criteria for patients enrolled in the study are shown.

Figure 2

In Figure 2a the linkage Disequilibrium structure between the haplotype tagging SNPs is depicted: rs3024718, rs3024731, rs3024772 are in tight linkage disequilibrium (LD). Figure 2b shows the four haplotypes defined by three tagging SNPs capturing 97% of the genetic variation for this single LD block within the entire PZ gene.
Figure 1: Flow chart of patient-parent trio selection

- **Electronic databases: ascertainment period 2000-2007**
  - Potentially relevant patients (first TE onset)
  - Aged neonate to ≤ 18 years
  - Σ = 633 [100%]

- **TE patients n = 460**
  - Excluded: Σ = 173 [27.3%]
    - Reasons for exclusion:
      - Lost to follow-up n = 37
      - No consent n = 17
      - Death n = 25
      - Vascular stroke n = 94

- **TE patients-parent trios n = 401**
  - Excluded: Σ = 59 [9.3%]
    - Reasons for exclusion:
      - Parents missing n = 59

- **Patients-parent trios included in the study Σ = 365 [57.7%]**
  - Non-vascular stroke n = 216
  - Venous thromboembolism n = 149
Figure 2: Linkage Disequilibrium structure (a) and haplotypes (b) in PZ genes
Table 1
Distribution of htSNPs in pediatric trios with non-vascular stroke (n=216) and thromboembolism (n=149)

<table>
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<th>htSNP* ID#</th>
<th>rs3024718</th>
<th>rs3024731</th>
<th>rs3024772</th>
<th>rs3024778</th>
<th>χ²</th>
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*htSNP: haplotype tagging single nucleotide polymorphism
Table 2:

Single point association (TDT) between variants in PZ and non-vascular stroke, TE, and combined data are shown.

<table>
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<tr>
<th>htSNP ID#</th>
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<th>χ²</th>
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<td>G</td>
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* T:U: ratio of transmissions to non-transmissions of the over-transmitted allele
Table 3: Association between PZ haplotypes and non-vascular stroke, TE, and combined data are shown.

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<th>Block</th>
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<th>χ²</th>
<th>P-Value</th>
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<td>AT</td>
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Association of the protein Z ATG haplotype with symptomatic non-vascular stroke or thromboembolism in white children - a family-based cohort study

Ulrike Nowak-Gottl, Birgit Frohlich, Sabine Thedieck, Andreas Huge and Monika Stoll