Reproducibility of Plasma Alkylresorcinols during a 6-Week Rye Intervention Study in Men with Prostate Cancer

Rikard Landberg, Afaf Kamal-Eldin, Swen-Olof Andersson, Jan-Erik Johansson, Jie-Xian Zhang, Göran Hallmans, and Per Åman

Abstract

Alkylresorcinols (AR), phenolic lipids exclusively present in the outer parts of wheat and rye grains, have been proposed as concentration biomarkers of whole-grain wheat and rye intake. A key feature of a good biomarker is high reproducibility, which indicates how accurately a single sample reflects the true mean biomarker concentration caused by a certain intake. In this study, the short- to medium-term reproducibility of plasma AR was determined using samples from a crossover intervention study, where men with prostate cancer (n = 17) were fed rye whole-grain/bran or refined wheat products for 6-wk periods. AR homologs C17:0 and C21:0 differed between the treatments (P < 0.001). The reproducibility determined by the intraclass correlation coefficient (ICC) was high (intervention period 1: ICC = 0.90 [95% CI = 0.82–0.98], intervention period 2: ICC = 0.88 [95% CI = 0.78–0.98]). The results show that a single fasting plasma sample could be used to estimate the mean plasma AR concentration during a 6-wk intervention period with constant intake at a precision of ± 20% (80% CI). This suggests that the plasma AR concentration can be used as a reliable short- to medium-term biomarker for whole-grain wheat and rye under intervention conditions where intake is kept constant.

Introduction

Alkylresorcinols (AR) (1,3-dihydroxy-5-alkylbenzene derivatives) are phenolic lipids found in high concentrations exclusively in the outer parts of wheat and rye grains among commonly consumed foods. The most common derivatives in wheat and rye are AR homologs with an odd carbon number in the alkyl chain (usually in the range of C17:0–C25:0). The AR C17:0/C21:0 ratio has been shown to reflect the source of whole-grain products in foods, with a ratio of 1.0, 0.1, and 0.01 indicating rye, common wheat, and durum wheat, respectively (1,2). AR in plasma have been proposed as a concentration biomarker of whole-grain intake and the plasma C17:0/C21:0 ratio has been suggested as a tool to differentiate wheat and rye intake (3–5). AR are absorbed to a great extent (~60%) and delivered to the lymphatic system in the upper gastrointestinal tract and transported in the blood mainly in lipoprotein fractions and in erythrocyte membranes (3,6,7). AR have been detected in adipose tissue of rats, suggesting that they, like many other lipophilic substances, are accumulated in adipose tissue (3,8–10). The elimination of AR is mainly through hepatic metabolism and subsequent urinary excretion (55–85% of a given dose) of 2 polar metabolites [3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-propanoic acid] (11). Both daily metabolite excretion and AR concentration in fasting plasma samples have been shown to increase in a dose-dependent manner after ingestion of AR and to reflect recent intake (4–6,11).

No study, to our knowledge, has estimated plasma AR reproducibility during intervention or free-living conditions. The reproducibility determines the number of samples needed to reflect the mean AR concentration caused by the individual’s usual whole-grain intake and is determined by the frequency of individual intake, absorption of the biomarker from the intestine, and the half-life of the biomarker in plasma and various tissues (12). In practice, a high reproducibility means that few repeated samples (minimum 1 sample) are required to accurately reflect the participant’s usual whole-grain intake (13,14). A biomarker with poor reproducibility requires a large number of samples and can lead to biased estimates of biomarker-disease associations (15). The reproducibility can be determined by the intraclass correlation coefficient (ICC), which is defined as follows:

\[ ICC = \frac{\sum (x_{ij} - \bar{x}_{..})^2}{\sum (x_{ij} - \bar{x}_{..})^2 + \sum (\bar{x}_{ij} - \bar{x}_{..})^2} \]

Where:
- \( x_{ij} \) is the observation on the jth individual for the ith measurement.
- \( \bar{x}_{..} \) is the overall mean.
- \( \bar{x}_{ij} \) is the mean of the jth individual.

The reproducibility can be estimated using the above formula, where the numerator represents the between-individual variance and the denominator represents the total variance.
ICC = \sigma^2_B/(\sigma^2_B + \sigma^2_w), where \sigma^2_B is the between-subject variance and \sigma^2_w is the within-subject variance (16).

The utility of plasma AR as concentration biomarkers is characterized by high specificity, but no study, to our knowledge, has reported their short-term or long-term reproducibility. In this study, the aim was to investigate the effect of very high AR intakes on fasting plasma AR concentration and to assess short-term (6 wk) reproducibility under intervention conditions where intake was kept constant. In addition, the number of samples required to determine the individual’s mean fasting AR plasma concentration during the intervention periods were estimated.

Materials and Methods

Participants and design. This study formed part of a study examining the effect of a diet rich in rye whole-grain/bran on a prostate cancer biomarker, prostate specific antigen. Men (n = 17) with clinically diagnosed prostate cancers completed the study, which was carried out on an out-patient basis in a randomized non-blind crossover design with 2 6-wk intervention periods (rye whole-grain/bran or refined wheat) separated by a 2-wk washout period. All participants received both treatments and were nonsmokers and reported no use of dietary supplements or medications. Their age was 73.5 ± 4.6 y (mean ± SD) and their BMI was 27.5 ± 4.6 kg/m². Single blood samples were taken before and between treatment periods and 3 times during each treatment (after 2, 4, and 6 wk). The entire study protocol was approved by the local ethics committee in the Uppsala region.

Diet and AR intake. Participants were instructed to keep their habitual diet except for cereal and table spread products, which were replaced by products provided by the study (intervention foods). During each treatment, participants were asked to include intervention foods as part of their diet except for cereal and table spread products, which were replaced by products provided by the intervention foods during the intervention periods (WasaBrod and Lantmännen. Food products were specially designed for the purpose of this study with the aim of providing the same macronutrient intake (Tables 1 and 2). To obtain the same fiber content in intervention foods for both treatments, purified wheat cellulose (Vitacel) was added to the refined wheat products.

| TABLE 1 | Advised and estimated daily macronutrient intakes provided by the intervention foods during the rye whole-grain/bran and refined wheat product treatments^1^2 |
|----------------|-----------------------------------|----------------|----------------|----------------|
| Macronutrient | Rye whole-grain/bran product treatment | Advised intake | Estimated intake | Refined wheat product treatment | Advised intake | Estimated intake |
| Protein, g/d | 60 | 52 ± 6 | 59 | 49 ± 8 | 40 | 36 ± 5 | 39 | 34 ± 5 |
| Fat, g/d | 40 | 36 ± 5 | 39 | 34 ± 5 | 60 | 52 ± 6 | 59 | 49 ± 8 |
| Carbohydrate, ^3^ g/d | 150 | 134 ± 16 | 155 | 135 ± 23 | 150 | 134 ± 16 | 155 | 135 ± 23 |
| Dietary fiber, g/d | 88 | 77 ± 9 | 89 | 76 ± 12 | 88 | 77 ± 9 | 89 | 76 ± 12 |
| Energy intake, kJ/d | 5124 | 4519 ± 543 | 5111 | 4418 ± 689 | 5124 | 4519 ± 543 | 5111 | 4418 ± 689 |

^1^ Values are means ± SD, n = 17.
^2^ Correspons to the amount of a certain food product distributed minus the amount left after treatment.
^3^ Excluding dietary fiber.

Before the study and during each treatment, participants conducted 4-d weighed food records to check compliance and to allow calculation of nutrient and total energy intake. The reported difference between distributed and consumed intervention foods at the end of each treatment was used as an additional check of compliance. Nutrient and energy intake were calculated from intervention product labels and by using the food database from the Swedish National Food Administration (PC-Kost 1.99, SLV) and the software program MATs 4.05 (Rudans lådadata). All intervention foods were analyzed for total AR content and relative homolog composition by the methods described briefly below. Mean daily AR intakes during the intervention periods were estimated from advised intervention food intake “by disappearance” (the difference between the intervention foods distributed to participants and any remaining foods after treatment) and by self-reported 4-d weighed food records. AR intake from 4-d weighed food records was calculated by using data from intervention foods analyzed, and, in a few cases, matching reported products to a corresponding product analyzed before.

Blood sampling. Blood samples (in total 8 per participant) were collected as fasting samples before and after 2, 4, and 6 wk in each treatment period. Blood drawn from an antecubital vein was collected in EDTA-coated vacuum tubes and immediately centrifuged (2000 × g; 10 min at 4°C) to separate plasma and erythrocytes. Plasma samples were portioned into 2-mL cryotubes and stored at –80°C until analysis. Plasma samples were available for all individuals but one, for whom 2 samples during the wheat product intervention period were missing.

Analytical methods. In foods, AR homologs C17:0, C19:0, C21:0, C23:0, C25:0, their sum (total AR), and the AR C17:0/C21:0 ratio were determined quantitatively by a GC method described in detail elsewhere (17). In brief, samples were milled, extracted with hot 1-propanol:water mixture (3:1, v:v), and analyzed by GC without any further purification or derivatization using methyl behenate as internal standard. Samples were portioned into 2-mL cryotubes and stored at C until analysis. AR homologs C17:0, C19:0, C21:0, C23:0, C25:0, their sum (total AR), and the AR C17:0/C21:0 ratio were determined. Homologs C17:0, C19:0, C21:0, C23:0, C25:0, their sum (total AR), as well as the AR C17.0/C21.0 ratio were determined. In brief, 200 μL plasma was mixed with internal standard (AR C20:0, ResaChem; 15 μL, concentration 1 mg/L) and extracted with diethyl ether (3 × 3 mL). Extracts were combined, evaporated, re-suspended in methanol (1 mL), and purified on Oasis-MAX solid phase extraction cartridges. Eluted AR were derivatized to trimethylsilyl-ethers and analyzed by GC-MS in SIM-mode. All samples were analyzed as single samples. Based on 5 control samples included in every batch, the
Results

All participants completed the study and complied well with the advised foods, as confirmed by agreement between the advised intervention food intake and the actual intake, determined as the difference between provided and reported remaining food products, and by food records (Tables 1, 2, and 3). The daily intake of total AR estimated from food records was $552 \pm 65$ mg/d during the rye whole-grain/bran treatment and $8.2 \pm 2.0$ mg/d during the refined wheat product treatment (Table 3). Energy intake did not differ before and after treatments and participants remained weight stable throughout the study (data not shown). The total AR plasma concentration was $991 \pm 794$ nmol/L during the rye whole-grain/bran product treatment and $75 \pm 92$ nmol/L during the refined wheat product treatment (Table 4). Plasma total AR concentration was higher at washout and during the refined wheat product treatment period for participants starting with rye whole-grain/bran products (Fig. 1C) and there were carryover effects ($P < 0.10$) for plasma C19:0, C21:0, C23:0, and for total AR concentrations. Due to the carryover effect, only plasma AR concentrations from the first study period were compared for evaluation of the treatment effect for these variables. Despite this, the plasma total AR concentration during the rye whole-grain/bran product treatment differed from that during the refined wheat product treatment ($P < 0.001$). There was no carryover effect for plasma AR homologs C17:0 and C25:0, which were both in higher concentration during the rye whole-grain/bran product treatment compared with the refined wheat product treatment ($P < 0.0001$). The AR C17:0/C21:0 ratio was higher ($P < 0.0001$) during the rye whole-grain/bran product treatment ($0.65 \pm 0.24$) than during the refined wheat product treatment ($0.27 \pm 0.22$) (Table 4; Fig. 1D).

The within-subject variations of plasma AR concentration were estimated to be 11 and 9% (in the range 4–47%) and the between-subject variations 33 and 25%, for the 2 study periods, respectively (Table 5). The ICC was $>0.85$ for both study periods with narrow 95% CI (Table 5). Only 1 sample was needed to estimate the steady-state plasma total AR concentration in these participants on constant whole-grain/bran intake with a precision of 20% at the confidence level of 80% and 2 samples are needed at the 95% confidence level (Table 5). For higher precision (5–10%), 2–19 samples are needed at the same confidence levels and the estimated number of samples required was slightly different between the 2 study periods (Table 5).

Discussion

We previously showed that plasma AR can serve as short- to medium-term concentration biomarkers for whole-grain wheat and rye intake (5,6,11). In the present study, plasma AR concentrations were analyzed 3 times during 6-wk intervention periods of rye whole-grain/bran or refined wheat products in men with prostate cancer to determine the short-term reproducibility of plasma AR concentrations in participants with constant intake.

As expected, plasma AR increased during the 6-wk rye whole-grain/bran intervention ($P < 0.001$), reaching an apparent steady state after 2 wk of intervention (Fig. 1), as evidenced by a short half-life of 4–5 h and regular intake (11,21). Higher plasma AR concentrations in the refined wheat product treatment of the group starting with rye whole-grain/bran products compared with the group starting with refined wheat products (Fig. 1C) explained the significant carryover effect observed for some AR homologs (C19:0, C21:0, and C23:0) and of plasma

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total AR intake, mg/d</th>
<th>AR C17:0/C21:0 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advised</td>
<td>Estimated by disappearance 2</td>
<td>Estimated by weighed 4-d records</td>
</tr>
<tr>
<td>Rye whole-grain/bran</td>
<td>620 (552 ± 65) [375–622]</td>
<td>550 (566 ± 160) [143–834]</td>
</tr>
<tr>
<td>Refined wheat</td>
<td>8.1 (7.7 ± 1.3) [4.4–9.0]</td>
<td>8.3 (8.2 ± 2.0) [3.2–13.2]</td>
</tr>
</tbody>
</table>

1 Values are median, (mean ± SD), and [range], n = 17.
2 Disappearance corresponds to the amount of a certain food product distributed minus the reported amount left after intervention period.
total AR concentration. This implies that AR were accumulated in different tissues representing various AR pools with a slower turnover rate during the rye whole-grain/bran product treatment and then subsequently released back to the plasma during the refined wheat product treatment. A 2-wk washout period is thus too short to completely empty pools of accumulated AR. The higher plasma AR concentration during refined product treatment in the second period could also have been due to inadequate compliance, but this was not supported by food records, which showed good compliance (Table 1; Fig. 1A).

The plasma AR C17:0/C21:0 ratio in the present study differed between treatments (P < 0.0001), which is consistent with previous results (4–6). Linko-Pavinen (7) suggested that a high plasma AR C17:0/C21:0 ratio (>0.6) together with a high plasma AR concentration (>200 nmol/L) indicates a major intake of whole grain from rye, whereas a low ratio (<0.2) in combination with a low plasma AR concentration (~33 nmol/L) is indicative of high intake of refined wheat. In principle, this statement is confirmed by the results of the present study, where the C17:0/C21:0 intake ratio was higher in the whole-grain/bran rye diet (~1.1) and slightly lower in the refined wheat diet (~0.15) compared with the ratios in plasma after consumption of corresponding diets (0.65 and 0.19, respectively), suggesting that nondietary factors such as differences in AR homolog pharmacokinetics seem to affect the plasma ratio (21). These factors need to be explored more before the proportion of consumed whole grains from different sources can be estimated from the AR C17:0/C21:0 ratio in plasma.

Simulation of plasma AR concentration based on a model (11) fitted to single-dose pharmacokinetic data (21) suggested considerable fluctuation in plasma AR concentration at steady state when consuming AR 3 times daily because of the short half-life (4–5 h) (data not shown). This suggests that time since the last whole-grain meal and frequency of intake are critical factors affecting the within-subject variation in AR plasma concentration and hence the possibility to accurately estimate the

### TABLE 4 Total AR concentrations and AR C17:0/C21:0 homolog ratios in plasma at baseline and during rye whole-grain/bran and refined wheat treatment periods1,2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rye whole-grain/bran</th>
<th>Refined wheat</th>
<th>AR C17:0/C21:0 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>78 (91 ± 67) [18–251]</td>
<td>73 (140 ± 73) [29.8–299]</td>
<td>0.36 (0.39 ± 0.12) [0.22–0.65]</td>
</tr>
<tr>
<td>2 wk</td>
<td>953 (885 ± 391) [167–1650]</td>
<td>47 (99 ± 120) [20–488]</td>
<td>0.63 (0.66 ± 0.23) [0.20–1.06]</td>
</tr>
<tr>
<td>4 wk</td>
<td>881 (909 ± 469) [288–2242]</td>
<td>42 (60 ± 45) [19–156.6]</td>
<td>0.69 (0.64 ± 0.26) [0.12–1.19]</td>
</tr>
<tr>
<td>6 wk</td>
<td>945 (1159 ± 1221) [261.1–5681]</td>
<td>33 (72 ± 101) [17–410]</td>
<td>0.70 (0.66 ± 0.25) [0.18–1.15]</td>
</tr>
<tr>
<td>Mean3</td>
<td>911 (991 ± 794) [167–5682]</td>
<td>40 (75 ± 92) [17–410]</td>
<td>0.66 (0.65 ± 0.24) [0.12–1.19]</td>
</tr>
</tbody>
</table>

1 Values are median, (mean ± SD), and [range], n = 17. Plasma samples were analyzed as single measurements.
2 Plasma AR concentration and C17:0/C21:0 were determined after 2, 4, and 6 wk within each treatment and mean values were calculated for each treatment.
3 Difference (P < 0.0001) between treatments as evaluated (using the means) by a general linear model appropriate for crossover design are indicated by different letters. Due to a carryover effect (P < 0.100), treatment effect of mean plasma total AR concentration was analyzed in parallel group design and differences (P < 0.001) are indicated by different letters.

### FIGURE 1 Dietary AR intake (|A|), C17:0/C21:0 intake ratio (|B|) estimated by 4-d weighed food records, plasma total AR concentration (|C|), and plasma C17:0/C21:0 ratio (|D|) in men with prostate cancer during rye whole-grain/bran and refined wheat treatment periods. Values are means ± SD, n = 17. Black triangle, group starting with the rye whole-grain/bran product treatment; white circle, group starting with the refined wheat product treatment. Food records 1–4, record day anytime within treatment period 1; records 5–8, record day anytime within treatment period 2. BL, Baseline; 2, 4, 6 wk, wk 2, 4, 6 of treatment; WO, washout period. Different letters indicate difference, P < 0.001, between treatment means (based on values reported within brackets), as evaluated in parallel design, due to a carryover effect, P < 0.100 (|C|). Different letters indicate difference, P < 0.0001, between the treatment means (based on values reported within brackets) as evaluated by an ANOVA model suitable for crossover designs (|D|).
TABLE 5  Estimated number of replicate samples needed from an individual to reflect the mean plasma total AR steady-state concentration corresponding to a certain intake during a 6-wk intervention period.

<table>
<thead>
<tr>
<th>D-level</th>
<th>First intervention period</th>
<th>Second intervention period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>8 (7.7, 8.3)</td>
<td>19 (18.7, 19.3)</td>
</tr>
<tr>
<td>10</td>
<td>2 (1.5, 2.5)</td>
<td>5 (4.5, 5.5)</td>
</tr>
<tr>
<td>20</td>
<td>1 (0.3, 1.7)</td>
<td>2 (1.2, 2.8)</td>
</tr>
<tr>
<td>30</td>
<td>1 (0.3, 1.7)</td>
<td>1 (–0.1, 2.2)</td>
</tr>
<tr>
<td>40</td>
<td>1 (0.3, 1.7)</td>
<td>1 (–0.1, 2.2)</td>
</tr>
<tr>
<td>50</td>
<td>1 (0.3, 1.7)</td>
<td>1 (–0.1, 2.2)</td>
</tr>
<tr>
<td>75</td>
<td>1 (0.3, 1.7)</td>
<td>1 (–0.1, 2.2)</td>
</tr>
<tr>
<td>90</td>
<td>1 (0.3, 1.7)</td>
<td>1 (–0.1, 2.2)</td>
</tr>
<tr>
<td>Within-subject CV%</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Between-subject CV%</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>ICC4</td>
<td>0.90 (0.82, 0.98)</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are point estimates (CI), n = 17.
2 CI for the estimated number of samples needed to reflect the basal AR level with precision D.
3 CI for the number of samples needed to measure the correct estimate, because the estimate for the second period might be partly biased by the carryover effect.
4 ICC defined as $(1 - r_{bs}^2)/(1 - r_{bs}^2 + r_{ws}^2)$, where $r_{bs}$ is the between-subject variance and $r_{ws}$ is the within-subject variance. ICC is presented with the corresponding 95% CI, given within parenthesis. Variance components were estimated by a mixed linear model in SAS v 9.1 (SAS Institute).

habitual intake of a participant. However, the present study showed that the within-subject variation was small (estimated at 9 and 11% for the respective periods) despite the apparent short elimination half-life for plasma AR. As a result, estimated ICC values were high for both periods. The low within-subject variation found in the present study is probably a result of the frequent and regular whole-grain intake (>3 times daily) and it appears that regular daily whole-grain intake compensates for the short half-life and gives high reproducibility in fasting plasma AR concentrations, which is a prerequisite for a reliable biomarker (12).

As expected, the between-subject variation in plasma AR concentration (~30%), which reflects differences in intake, absorption, distribution, and elimination, was higher than the within-subject variation (~10%). Both within- and between-subject variation in the present study were lower compared with what was estimated for enterolactone (ENL) (suggested as a biomarker of plant food intake) under intervention conditions with constant intake (14,22) and in free living participants (23,24). In a 3-way crossover study by Kuijsten et al. (22) where foods with controlled lignan content were given 2 times/d during 10-d long treatment periods, the between- and within-subject variation of ENL was estimated at 46–95% and 35–49%, respectively, somewhat depending on the lignin bioavailability in administrated foods. In another intervention study with constant lignan intake 3 times/d for 3 d, the between- and within-subject variance components were estimated at 72 and 56%, respectively (14). The lower between-subject variation observed for AR is probably due to the fact that there are only a limited number of significant determinants for plasma AR concentration (R. Landberg, A. Olsen, A. Kamal-Eldin, P. Åman, and A. Tjønneland, unpublished data), whereas a number of dietary and nondietary factors determine plasma ENL concentration (25,26). The low within-subject-between-subject variance ratio resulted in somewhat higher ICC compared with what was estimated for ENL under intervention conditions (14). Due to the high ICC, only 1–2 fasting plasma samples were needed to estimate the mean plasma AR concentration of a 6-wk period with constant intake with 20% precision at a confidence level of 80–95%. For ENL under intervention conditions with constant intake, 5 repeated samples were calculated to be necessary to estimate an individual’s mean serum concentration with 50% precision at a CI level of 95% (14). Two studies in free-living participants estimated that 3 repeated samples were needed to asses the mean ENL concentration with the same precision but at the CI level of 80% (13,24). Because a significant carryover effect was observed for some AR homologs in the present study, the ICC determination for the first period is probably a more correct estimate, because the estimate for the second period might be partly biased by the carryover effect.

Under conditions similar to those in the present study and using the variance components estimated, any observed association between AR concentration determined by a single sample and endpoint (or variable measured without measurement error) would be underestimated by 6%. To compensate for this underestimation caused by the error of using a single biomarker measurement, the number of participants would need to be increased by 12% as calculated by formulae given in the literature (16,27,28).

The reproducibility shown in this intervention study with constant intake was high and is expected to be lower in a free-living population where the variation in AR intake is larger. However, AR are typically found in food items consumed regularly, such as bread and breakfast cereals. As highlighted by van Dam and Hu (29), the long-term reproducibility in population-based material remains to be determined to evaluate the accuracy of plasma AR concentrations as biomarkers for whole-grain wheat and rye intake and their usefulness in ranking the free-living population on the basis of whole-grain wheat and rye intake in epidemiological studies. When the long-term reproducibility of the biomarker has been estimated, it could be used to compensate for attenuation in relations between biomarker measurement and estimated disease risk caused by the misclassification inherent in the measurement error of a single biomarker measurement (30).

In summary, measurement of plasma AR concentrations showed excellent reproducibility under intervention conditions, i.e. when the AR intake was controlled and kept constant. One fasting sample reflected the mean short-term (6-wk) concentration of the biomarker. However, the reproducibility of plasma AR as biomarkers of whole-grain wheat and rye intake in free-living individuals, where the intake is expected to vary between days, remains to be determined.

Acknowledgments
We thank Erika Jansson for her skilled technical assistance and Gerd Berglund for her skilled support during the practical phase of the experiment. We thank Lars Berglund and Sylvia Olofsson at Uppsala Clinical Research Centre for statistical consultation.

Literature Cited