Novel somatic mutations and distinct molecular signature in aldosterone-producing adenomas

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Abstract

Aldosterone-producing adenomas (APAs) are found in 1.5–3.0% of hypertensive patients in primary care and can be cured by surgery. Elucidation of genetic events may improve our understanding of these tumors and ultimately improve patient care. Approximately 40% of APAs harbor a missense mutation in the KCNJ5 gene. More recently, somatic mutations in CACNA1D, ATP1A1 and ATP2B3, also important for membrane potential/intracellular Ca2+ regulation, were observed in APAs. In this study, we analyzed 165 APAs for mutations in selected regions of these genes. We then correlated mutational findings with clinical and molecular phenotype using transcriptome analysis, immunohistochemistry and semiquantitative PCR. Somatic mutations in CACNA1D in 3.0% (one novel mutation), ATP1A1 in 6.1% (six novel mutations) and ATP2B3 in 3.0% (two novel mutations) were detected. All observed mutations were located in previously described hotspot regions. Patients with tumors harboring mutations in CACNA1D, ATP1A1 and ATP2B3 were operated at an older age, were more often male and had tumors that were smaller than those in patients with KCNJ5 mutated tumors. Microarray transcriptome analysis segregated KCNJ5 mutated tumors from ATP1A1/ATP2B3 mutated tumors and those without mutation. We observed significant transcription upregulation of CYP11B2, as well as the previously described glomerulosa-specific gene NPNT, in ATP1A1/ATP2B3 mutated tumors compared to KCNJ5 mutated tumors. In summary, we describe novel somatic mutations in proteins regulating the membrane potential/intracellular Ca2+ levels, and also a distinct mRNA and clinical signature, dependent on genetic alteration.

Key Words

- ATP1A1
- CACNA1D
- KCNJ5
- primary aldosteronism
- aldosterone-producing adenoma

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Introduction

Primary aldosteronism (PA) is the most common endocrine cause of secondary hypertension with an estimated prevalence of ~5–10% in unreferral hypertensive patients and up to 20% of those with grade III hypertension (Rossi et al. 2006, Fogari et al. 2007, Hannemann & Wallaschofski 2012). Aldosterone-producing adenomas (APAs) represent a surgically curable form of PA and occur in ~30% of PA patients (Rossi et al. 2006, Fogari et al. 2007). The aldosterone overproduction leads to increased Na⁺ and H₂O retention by the kidney, resulting in hypertension. Importantly, the excess aldosterone also promotes cardiac remodeling and vascular collagen deposition, augmenting cardiovascular disease in these patients when compared to patients with essential hypertension (Brilla & Weber 1992, Rossi et al. 1997, Savard et al. 2013).

In the normal zona glomerulosa (ZG) cell, the plasma membrane is kept at a highly hyperpolarized state (Quinn et al. 1987). This hyperpolarization is mainly attributed to a dominant conductance of potassium ions over the cell membrane (Spät & Hunyady 2004). Depolarizing the membrane potential increases intracellular Ca²⁺ and subsequently increases aldosterone production and secretion (Spät & Hunyady 2004).

Choi et al. (2011) identified mutations in the KCNJ5 gene in both sporadic APAs and in a family with bilateral adrenal hyperplasia. Mutations affecting the selectivity filter of GIRK4 (encoded by the KCNJ5 gene) resulted in the loss of selectivity for potassium, increased sodium influx and subsequent membrane depolarization. The membrane depolarization raises intracellular calcium concentrations, leading to increased CYP11B2 transcription and subsequent aldosterone production (Oki et al. 2012). Multiple screening studies of over 1000 sporadic APAs have established a prevalence of ~40% mutations in KCNJ5 in Western countries (Akerstrom et al. 2012, Boulkroun et al. 2012, Lenzini et al. 2015) and even higher in Asian countries (Taguchi et al. 2012, Kitamoto et al. 2015, Wang et al. 2015, Zheng et al. 2015).

More recently, somatic mutations in genes encoding an additional ion channel (CACNA1D) and two ATPases (ATP1A1 and ATP2B3) were discovered in APAs (Azizan et al. 2013, Beuschlein et al. 2013, Scholl et al. 2013). Also, germline mutations in CACNA1D and CACNA1H were described in patients with early onset PA (Scholl et al. 2013, Scholl et al. 2015). Similar to GIRK4, the protein products of these genes are ultimately involved in regulating the membrane potential and Ca²⁺ homeostasis in adrenal glomerulosa cells. To provide better knowledge of prerequisites for potential diagnostic and therapeutic targets, we aimed to investigate the frequency of mutations in CACNA1D, ATP1A1 and ATP2B3 in 165 APAs, and explore clinical and molecular phenotype.

Materials and methods

Included patients and clinical annotation

Tumor specimens were collected from 165 patients (65 males and 100 females) with diagnosed APA from six different tertiary referral centers. The diagnosis was established by raised aldosterone/renin ratio, positive confirmatory tests, lateralization studies (AVS and/or CT/MRI) and histopathological examination, according to routine protocols at the individual centers. Patients gave written informed consent and approvals from the local ethical committees were obtained.

Cortisol-producing adenomas (n=4) and non-secreting adenomas (n=4) were included for CYP11B2 expression analysis. Patients with cortisol-producing adenomas had elevated cortisol in serum and failure to suppress cortisol with dexamethasone and postoperative cure. Non-hormone secreting adenomas were detected incidentally during abdominal CT scan. These were deemed functionally silent after negative hormone analyses and were removed on suspicion of malignancy (size or other radiological signs).

Molecular genetic analyses

DNA/RNA was extracted using Allprep DNA/RNA mini kit, (Qiagen), AllPrep DNA/RNA FFPE Kit (Qiagen) or DNeasy Blood and tissue kit, (Qiagen). cDNA was prepared using Revertaid H-minus First strand cDNA synthesis Kit, (Fermentas, Waltham, MA, USA). In total, 90 (54.5%) KCNJ5 mutations had previously been observed (Akerstrom et al. 2012). Hotspot regions in CACNA1D exons 8A, 8B, 16 and 33, ATP1A1 exons 4, 8 and 21 and ATP2B3 exon 8 were analyzed in all tumors. In addition, tumors without KCNJ5 mutation (n=75) were analyzed for fragments corresponding to regions in CACNA1D exons 5, 6, 13, 14, 23, 27, 32, 35, 36, 37, 38, 39, 40. When available, confirmatory PCR was performed on DNA from peripheral blood/surrounding normal tissue as well as in cDNA. Details on PCR conditions and primer sequences are available upon request. Sanger sequencing was conducted on amplified fragments (Beckman Coulter Genomics, Takeley, UK). Chromatograms were analyzed...
using CodonCode aligner Software 4.2.4 (CodonCode Corporation, Dedham, MA, USA). NCBI Reference Sequences; NP_001122312.1 and NP_000711 (exon 8B) for \textit{CACNA1D}, NP_001153705.1 for \textit{ATP1A1} and NP_001001344.1 for \textit{ATP2B3} were used for sequence alignment. Evolutionary conservation was determined using the ClustalW2 algorithm (Larkin \textit{et al.} 2007).

Transcriptome analysis was performed on 12 APAs (six \textit{KCNJ5} mutated, three \textit{ATP1A1/2B3} mutated and three tumors without known mutation) using the SurePrint G3 Human Exon 4×180K Microarray (Agilent Technologies, Santa Clara, CA, USA). Data analysis was conducted using the GeneSpring Software v.13 (Agilent Technologies). Quality control of the included samples was performed in the GeneSpring v.13 Software using a flag-based method. Baseline transformation to the median of all samples was performed. Data were log-transformed and normalized using the quantile method. Unsupervised hierarchical clustering based on Euclidean distances and complete linkage was obtained. Heat maps of the top 100 up- and downregulated transcripts in \textit{ATP1A1/2B3} mutated tumors compared to \textit{KCNJ5} mutated tumors were generated and \textit{t}-tests were performed. Subsequently, the Benjamini-Hochberg method was used for multiple testing correction, and significantly differentially expressed transcripts were identified by volcano plots using fold change \textit{Z} as the criteria. If significant group effects were present, the overall group effect was analyzed using One-way ANOVA for parametric data and a Kruskal-Wallis test for non-parametric data. SPSS 22 (IBM) was used for statistical analysis.

The proportion of mutations and corresponding phenotypes of the screened cohorts are summarized in Tables 1 and 2 respectively. Tumors from three of the centers contained more than 50% \textit{KCNJ5} mutations (Table 1). These cohorts had relatively large tumors, with a mean size of 17.9 mm ±0.92, and included many female patients (68%), and most patients were hypokalemic (96%). For the entire cohort, we observed a female gender

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<thead>
<tr>
<th>Table 1</th>
<th>Proportion of mutations in APAs stratified by cohorts</th>
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<tbody>
<tr>
<td>Cohort</td>
<td>Number of APAs</td>
</tr>
<tr>
<td>Uppsala</td>
<td>36</td>
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<tr>
<td>Dusseldorf</td>
<td>33</td>
</tr>
<tr>
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<td>8</td>
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<tr>
<td>Lübeck</td>
<td>20</td>
</tr>
<tr>
<td>Sydney</td>
<td>34</td>
</tr>
<tr>
<td>Stockholm</td>
<td>34</td>
</tr>
<tr>
<td>Summary</td>
<td>165</td>
</tr>
</tbody>
</table>
overrepresentation for patients with KCNJ5 mutated tumors compared to patients without KCNJ5 mutated tumors (80% vs 38%, \( P < 0.0001 \)) (Table 2). These patients were also operated at a younger age (44.9 years ± 1.23 vs 53.1 years ± 1.34, \( P < 0.0001 \)). No difference in adenaoma size (19.4 mm ± 0.80 vs 16.5 mm ± 1.27, \( P = 0.17 \)) or preoperative aldosterone levels was observed (1365 pmol/l ± 219 vs 1355 pmol/l ± 145, \( P = 0.97 \)).

A total of 20 (12.1%) mutations in CACNA1D, ATP1A1 and ATP2B3 (Table 1) were identified. The somatic nature of the mutations was verified in all samples with available non-tumoral tissue (Supplementary Table 1, see section on supplementary data given at the end of this article). Mutations in CACNA1D were found in 3.0% of tumors. These mutations were located in exon 8A, 16 and 33, corresponding to segment 6 (S6) in repeats I and II, and S5 in repeat IV (Supplementary Fig. 1). We discovered a novel mutation in exon 8A (p.Val401Leu). Val401 is an evolutionary conserved amino acid located two positions upstream from the frequently mutated Gly403 residue (Supplementary Table 2). Somatic mutations in ATP1A1 were observed in 10/165 (6.1%) and in ATP2B3 in 5/165 (3.0%) APAs. The mutations in ATP1A1 comprised the previously described p.Leu104Arg (c.311T > C) along with three novel deletions in the same area, as well as three novel deletions in the M9 domain (Supplementary Figs 2A and 3, and Supplementary Table 3). The mutations found in ATP2B3 corresponded to amino acid residues located in the M4 domain of the protein. These included two previously described deletions and two novel deletions (Supplementary Figs 2B and 4). All of the observed mutations were found in non-KCNJ5 mutated tumors.

We observed a significant difference in clinical phenotype in patients with ATP1A1/2B3 and CACNA1D mutated tumors compared to patients with KCNJ5 mutated tumors (Table 2). ATP1A1/2B3 and CACNA1D mutated tumors were significantly smaller than KCNJ5 mutated APAs (11.2 mm ± 0.94 vs 19.4 mm ± 0.80, \( P < 0.0001 \)). Patients were operated at an older age (53.7 years ± 2.35 vs 44.9 years ± 1.23, \( P = 0.008 \)), and they were more often male (65% vs 20%, \( P < 0.0005 \)). No significant difference in preoperative aldosterone levels was observed (1671 pmol/l ± 340 vs 1365 pmol/l ± 219, \( P = 0.30 \)).

Transcriptome analysis and unsupervised hierarchical clustering using 10% of probes with the highest variation in expression segregated KCNJ5 mutated tumors (Fig. 1A). Likewise, principal component analysis on all probes segregated KCNJ5 mutated tumors (Fig. 2). Unsupervised hierarchical clustering on the complete set of probes segregated KCNJ5 mutated tumors, except for one
confirming higher expression in ATP1A1/2B3 mutated tumors ($P<0.05$) (Fig. 4). To verify increased NPNT expression in ATP1A1/2B3 tumors, we analyzed protein expression using immunohistochemistry. As expected, staining of normal kidney tissue revealed selective expression in glomeruli and blood vessels (Fig. 5A). In normal adrenal tissue, expression was restricted to ZG cells (Fig. 5B). Staining of tumor tissue confirmed array findings with higher expression of NPNT in ATP1A1/2B3 mutated APAs compared to KCNJ5 mutated APAs (Fig. 5C and D).

**Discussion**

In this study, we observed somatic mutations in CACNA1D, ATP1A1 and ATP2B3 in 12.1% of APAs, including nine novel somatic mutations. CACNA1D mutations were detected in 3% of the tumors. CACNA1D encodes the alpha-1 subunit of an L-type voltage-dependent Ca\(^{2+}\) channel (VDCC) important for increasing intracellular Ca\(^{2+}\) and aldosterone production in ZG cells (Cohen et al. 1988). The observed mutations were located in segment 6 in repeats I and II, and S5 in repeat IV (Fig. 6). Interaction between S5 and S6 is important for the normal function of the channel pore (Catterall 2010). *In vitro* studies have shown that alterations in these portions of the protein lead to increased channel activity (Azizan et al. 2013, Scholl et al. 2013). We detected a novel missense mutation (p.Val401Leu) in one of the tumors. This position is located two amino acid residues upstream from the Gly403 in S6 (repeat I), one of the most frequently mutated residues in CACNA1D (Azizan et al. 2013, Scholl et al. 2013, AB –10 0 10 –7.2 0 7.2
mutations have resulted in a gain of function effect in other VDCCs (Ducros et al. 2001, Pietrobon 2002, Hoda et al. 2005, Striessnig et al. 2010, Stockner & Koschak 2013). Interestingly, in two studies conducted in Asia, only two mutations in 282 analyzed APAs were found (Wang et al. 2015, Zheng et al. 2015). They also observed a high prevalence of KCNJ5 mutations, 76.2% (215/282). Similar to the Asian cohorts, our prevalence of KCNJ5 mutations was relatively high. Compared to another large European cohort of 474 APAs (Fernandes-Rosa et al. 2014), our patients were more often female and had larger adenomas, a phenotype associated with KCNJ5 mutations (Akerstrom et al. 2012, Azizan et al. 2012a, Fernandes-Rosa et al. 2014). This could potentially explain our relatively low number of CACNA1D mutations, which seem to occur in smaller tumors in male patients (Azizan et al. 2013, Scholl et al. 2013, Fernandes-Rosa et al. 2014). This affirms the notion that aside from a possible demographic bias, the diagnostic workup and selection of patients affect the distribution of mutations.

We detected ATP1A1 mutations in 6.1% of tumors. ATP1A1 encodes the alpha-1 subunit of a ubiquitously expressed Na⁺/K⁺ ATPase, vital for establishing the membrane potential in ZG cells (Spät & Hunyady 2004). The mutations were either located close to or involving amino acid Leu104 in the M1 domain, or affecting the amino acid Leu104 in the M1 domain, or affecting the membrane potential in ZG cells (Spät & Hunyady 2004).

Figure 3
Statistically significant expression differences in ATP1A1/2B3/WT compared to KCNJ5 mutated APA. Volcano plot on all quality passed probes on the microarray. The x-axis shows the fold change (log2) and the y-axis shows corrected P value (−log10). T-tests were performed on greater than twofold up/down regulated probes and correction for multiple testing by the Benjamini-Hochberg method was applied. Probes with a fold change >2 and a false discovery rate of <0.05 in (A) ATP1A1/2B3 mutated tumors compared to KCNJ5 mutated tumors and (B) WT tumors compared to KCNJ5 mutated tumors, displayed in red.

Figure 4
mRNA expression of CYP11B2 in ATP1A1/2B3 and KCNJ5 mutated adenomas. Relative mRNA expression (2−ΔΔCt) of CYP11B2 in ATP1A1 and ATP2B3 mutated APAs = ATPase compared to KCNJ5 mutated, NHSA and CPA. GAPDH was used as an internal control. The relative level of CYP11B2 was compared between ATPase and KCNJ5 mutated tumors using a Mann-Whitney’s U-test. Median and maximum values are displayed. NHSA, non-hormone secreting adenoma; CPA, cortisol producing adenoma; *P < 0.05. Insert depicts the individual levels of CYP11B2 in the ATP1A1/2B3 mutated tumors (median and range).
transmembrane helix M4, leading to potassium binding and occlusion by the channel (Einholm et al. 2007). P.Leu104Arg and p.Gly99Arg mutations likely disrupt this site, and have been shown to increase depolarization, CYP11B2 expression and aldosterone production in vitro (Azizan et al. 2013, Williams et al. 2014). The mutations in the M9 domain were novel deletions affecting amino acids Glu960 and Glu961 in the third Na\(^+\) binding site of the protein. Previous in vitro studies of a p.Glu961Ala mutant showed reduced Na\(^+\) binding and a lowered affinity for K\(^+\) (Li et al. 2005). Mutations in ATP1A1 have previously not been described in any syndromes, and no germline mutation could be detected in screened familial PA cases (Beuschlein et al. 2013). Given its ubiquitous expression and vital role for establishing the membrane potential, this is not surprising (Jorgensen et al. 2003). However, an association between ATP1A1 haplotypes and primary hypertension has been described (Herrera et al. 1998, Treva Rice et al. 2000, Glorioso et al. 2007). Further investigation of the functional consequences of ATP1A1 genotypes and haplotypes in hypertensive cohorts are therefore warranted.

ATP2B3 encodes a plasma membrane Ca\(^{2+}\) ATPase that actively transports Ca\(^{2+}\) out of the cell, resulting in low intracellular Ca\(^{2+}\) levels, thereby inhibiting aldosterone production (Spät & Hunyady 2004). We observed five APAs with ATP2B3 mutations, including two novel deletions and two previously reported deletions (Beuschlein et al. 2013, Williams et al. 2014). The mutations span a seven amino acid region in the M4 domain, a part of the protein highly conserved among orthologous. Importantly, these amino acids are located at similar positions as the mutated residues in the M4 domain in ATP1A1. In contrast to ATP1A1, a germline ATP2B3 mutation has been found in a family with X-linked congenital cerebellar atrophy (Zanna et al. 2012). Functional studies showed that this mutation (p.Gly1107Asp) decreased the ability of the channel to extrude Ca\(^{2+}\) from the cells. To our knowledge, hyperaldosteronism has not been observed in these patients.

Previous studies have described genotype-phenotype correlations in APAs. While some have not described any gender association (Scholl et al. 2013, Williams et al. 2014), we could confirm that ATP1A1/2B3/CACNA1D mutated tumors are more often found in male patients, compared to a female overrepresentation for KCNJ5 mutated tumors (Azizan et al. 2013, Beuschlein et al. 2013, Dutta et al. 2013, Fernandes-Rosa et al. 2014). We also observed an older age at the time of operation for patients with these tumors, previously reported by some (Azizan et al. 2013, Scholl et al. 2013, Fernandes-Rosa et al. 2014) but not by others (Beuschlein et al. 2013, Dutta et al. 2013, Williams et al. 2014). We could not confirm higher preoperative aldosterone levels (Beuschlein et al. 2013), but observed the previously reported association with smaller tumors (Azizan et al. 2013, Dutta et al. 2013, Scholl et al. 2013, Fernandes-Rosa et al. 2014). Importantly, due to their smaller size ATP1A1/2B3/CACNA1D mutated tumors may be missed in patients that have not undergone AVS, which may also lead to delayed diagnosis and subsequently an older age at the time of the operation. It also suggests that the use of only CT/MRI may bias the mutational spectrum towards more KCNJ5 mutations, as previously indicated (Fernandes-Rosa et al. 2014).

Previous reports have described a ZG-like histological appearance in ATP1A1/2B3/CACNA1D mutated tumors compared to a more ZF-like composition in KCNJ5 mutated tumors (Azizan et al. 2013, Dekkers et al. 2014, Monticone et al. 2015). Also, high CYP11B2 and low CYP11B1 protein expression have been observed in these APAs (Azizan et al. 2013, Dekkers et al. 2014, Monticone et al. 2015). However, a large transcriptomic analysis of
92 APAs did not detect any differences in CYP11B2 and CYP11B1 expression (Fernandes-Rosa et al. 2014). Neither did it detect a specific transcriptome signature (Boulkroun et al. 2012) nor a specific histological appearance (Fernandes-Rosa et al. 2014). Our transcriptomic analysis revealed differences between KCNJ5 mutated APAs and the other tumors. However, because of the low number of samples, our results should be interpreted with some consideration. Interestingly, of the two transcripts displaying higher expression in our ATP1A1/2B3 mutated tumors, NPNT was previously described as a ZG marker and the second most upregulated transcript in ATP1A1/CACNA1D mutated tumors compared to KCNJ5 mutated tumors (Azizan et al. 2013). NPNT encodes nephronectin, an extracellular matrix protein important for kidney development (Linton et al. 2007). Immuno-histochemistry verified nephronectin as a possible ZG cell marker and confirmed higher expression in ATP1A1/2B3 mutated tumors compared to KCNJ5 mutated tumors. We also confirmed previous reports of higher CYP11B2 expression in our ATP1A1/2B3 mutated tumors. The lower CYP11B2 expression in APAs harboring KCNJ5 mutations may indicate a relatively lower production of aldosterone per cell, possibly compensated by their larger size, explaining the similar serum levels of aldosterone in patients with ATP1A1/2B3/CACNA1D mutated tumors. Our results provide additional evidence that KCNJ5 mutated APAs do display subtle differences in their molecular phenotype compared to ATP1A1/2B3 mutated tumors (Azizan et al. 2012b, Azizan et al. 2013, Dekkers et al. 2014, Monticone et al. 2015). Perhaps these small variances reflect a different cellular origin of KCNJ5 mutated APAs, but because of similar intracellular events (i.e., increased Ca$^{2+}$) these remain subtle. Of note, most KCNJ5 mutated APAs express markers of ZG cells such as DAB2 (Boulkroun et al. 2010), making the notion of different cellular origins more complex. A limitation of this study was that we re-sequenced selected regions of these genes, possibly lowering the true prevalence of mutations. The entire coding segments of ATP1A1 and ATP2B3 have previously been analyzed in a large cohort of APAs (Beuschlein et al. 2013), and the results suggest that mutations outside these areas are rare. Mutations in CACNA1D have been found in hotspot areas in multiple exons. We chose to sequence all areas in CACNA1D where mutations previously have been described in APAs, and also included areas where gain of function mutations have been observed in other disorders. We could not detect any mutations outside mutational hotspot areas. If a gain of function effect is the cause of these tumors, the chance of finding other prevalent mutational areas seems less likely but cannot be excluded. Also, we did not perform functional characterization of our novel mutations; however, due to the conservation in different organisms and their location, we infer an altered protein function.

In conclusion, somatic and germline mutations in regulators of the membrane potential and/or intracellular Ca$^{2+}$ homeostasis occur frequently in APAs. Also, non mutated tumors display a similar mRNA signature as ATP1A1/2B3 mutated tumors. This makes it tempting to speculate that additional proteins involved in this
regulatory pathway are important for the tumorigenesis. With improved understanding of these events, the possibility of more specific treatment and better diagnostics will increase.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0321.

Declaration of interests
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Author contributions: T Åkerström performed experiments, carried out statistical analysis and drafted the manuscript. T Åkerström, S Backman, H S Willenberg, K Cupisti, J Ip, A Moser, R Maharjan, B Robinson, K A Iven, H Drale, C D Volpe, M Bäckdahl, J Botling, P Li, G Westin, M K Walz, H Lehner, S Sidhu, J Zedenius, P Björklund and P Hellman participated in either the acquisition, analysis, or interpretation of data. All authors contributed with critical revision of the manuscript and all authors performed administrative, technical or material support. P Björklund and P Hellman obtained funding. T Åkerström and P Björklund designed the study. P Björklund supervised the study.

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