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Original Experimental

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Circulating long non-coding RNA signature in knee osteoarthritis patients with postoperative pain one-year after total knee replacement

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Abstract

Objectives: The incidence of chronic postoperative pain after total knee replacement (TKR) is approx. 20%, and hence preoperative risk factors are important to identify. Recent studies have indicated that preoperative inflammatory markers might hold prognostic information for the development of chronic postoperative pain. Long non-coding RNA (lncRNA) regulates the expression of genes related to e.g. inflammatory processes. The current study aimed to investigate the preoperative lncRNA signature as possible preoperative predictive markers for chronic postoperative pain following TKR.
Methods: Serum samples, collected preoperatively from 20 knee osteoarthritis (KOA) patients, were analyzed for 84 validated circulatory lncRNA. Pain intensity was assessed using a visual analog scale (VAS) before and one-year after TKR. Differences for the lncRNA expression were analyzed between patients with chronic postoperative pain (VAS≥3) and those with a normal postoperative recovery (VAS<3).
Results: LncRNA *Myeloid Zinc Finger 1 Antisense RNA 1 (MZF1-AS1)* (fold change −3.99; p-value: 0.038) (shown to be involved neuropathic pain) *Metastasis associated lung adenocarcinoma transcript 1 (MALAT1)* (fold change −3.39; p-value: 0.044) (shown to be involved neuropathic pain); *Patched 1* pseudogene *(LOC100287846)* (fold change −6.99; p-value: 0.029) (unknown in pain) were down-regulated preoperatively in the group with chronic postoperative pain compared to the group normal postoperative pain recovery.
Conclusions: These findings suggest, that TKR patients with chronic postoperative pain present preoperative downregulations of three specific lncRNA detectable at the systemic level. The presented study might give new insights into the complexity of chronic postoperative pain development and show how non-coding RNA plays a role in the underlying molecular mechanisms of pain.

Keywords: circulating pain biomarker; knee osteoarthritis; long non-coding RNA; pain.

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Introduction

Osteoarthritis (OA) is one of the most frequent painful musculoskeletal diagnoses in the elderly population and the most prominent cause of disability [1]. Total knee replacement (TKR) is the end-stage treatment of knee OA and provides pain relief for most patients with severe OA. Unfortunately, around 20% of knee OA patients will

experience chronic postoperative pain after an otherwise technically successful TKR [2, 3]. Studies have shown that epigenetic modifications, such as DNA methylation, histone modifications and the actions of non-coding RNA (ncRNA), may confer susceptibility to OA, which could open up new avenues for alternative therapeutic approaches [4, 5]. Several studies have illustrated the diagnostic potential of circulating ncRNA in the synovial fluid of patients with OA [6], indicating that circulating microRNA (miRNA) could be potential predictors for severe knee and hip osteoarthritis [7]. Despite many studies highlighting different functions of miRNA involved in various pain states, long non-coding RNA (lncRNA) [8], ncRNA of >200 nucleotides in length, are still to be investigated for the roles in the development of painful conditions. Biological activities of lncRNA are still being elucidated, but studies have shown their tissue-specific expression pattern in humans [9] and their involvement in regulating chromatin modification, gene transcription, miRNA translation and regulation, and protein function [10–12]. Circulating lncRNA have been detected in several body fluids [13], and in extracellular vesicles (i.e. exosomes), which likely increase their half-life *in vivo*, and thus their potential as a molecular biomarker [14]. For this reason, these molecules are increasingly utilized as biomarkers for the development of cancer [15–17] but have been less explored on pain. A recent study indicates that more than 50% of the lncRNA are expressed in the central nervous system [18], indicating the functional importance of lncRNA in brain disorders such as Multiple Sclerosis [19]. A pre-clinical study addressed an association between lncRNA and nociceptive modulation and identified a novel antisense RNA (asRNA) lncRNA for the potassium channel Kcna2 in rat dorsal root ganglion (DRG) [20]. Moreover, Fu and colleagues showed variable expression of lncRNA in the cartilage of OA patients, demonstrating upregulation of a specific lncRNA, *uc.343*, that acts on genes implicated in the development of cartilage, leading to a cartilage disruption and faster progression of OA [21]. In a human experimental phasic pain model two lncRNA, such as ZNRD1-AS and RP11-819C21.1, were up-regulated and directly correlated with features of the laser evoked potential suggesting that lncRNA could be involved in abnormal excitability of the cerebral cortex in relation to pain [22].

The aim of this study was to investigate the preoperative expression of 84 circulating lncRNA related to the regulation pro-/anti-inflammatory genes and microRNAs expression, as a potential preoperative signature for the development of chronic postoperative pain one-year after TKR.

Materials and methods

Patients

Twenty patients with Knee Osteoarthritis (KOA) scheduled for total knee replacement (TKR) were recruited from the outpatient clinic at Hospital Vendsyssel, Frederikshavn, Denmark. Patients with other diagnosed pain condition (e.g., hip OA, rheumatoid arthritis, fibromyalgia, and neuropathic pain), or mental impairment were excluded from the study. Radiological KOA was evaluated using the Kellgren and Lawrence (KL) score [23]. The patients were asked not to take any analgesic medication 24 h before the preoperative study visit. The study was approved by The North Denmark Region Committee on Health Research Ethics (N-20120015) and conducted in accordance with the Helsinki Declaration. All patients read and signed an informed consent form prior to enrollment.

Pain assessment

The peak pain intensities within the last 24 h were collected before surgery and one year after.

The participants were asked to rate their pain intensity on a 10 cm scale from 0 to 10 where “0” represents “no pain” and “10” represents “worst pain imaginable” (visual analog scale [VAS]). The sample presented in the current manuscript is a subpopulation obtained from a larger study from which the patients were chosen and divided into two groups based on their postoperative pain intensity.

Patients with postoperative VAS=10 were considered patients with chronic postoperative pain and assigned to the “Pain” group, whereas patients with postoperative VAS=0 were considered as having a normal postoperative pain recovery and assigned to the “No pain” group.

Blood sampling and serum isolation

Venous blood was collected following standard venipuncture procedures, between 07:30 and 09:00 in the morning on the day of surgery. Nine milli-liter of whole blood was withdrawn in an untreated tube. After collection, the whole blood was left for 15 min at room temperature to allow the coagulation. The coagulated blood was removed centrifuging at 3,000 RPM for 15 min and the serum obtained was transferred into a clean tube and stored at −80 °C until used. After thawing on ice, total RNA was isolated from 200 µL of serum using miRNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Long non-coding detection

Total RNA extracted was subjected to complementary DNA (cDNA) synthesis using RT^2 PreAMP cDNA Synthesis Kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA samples were preamplified using Human RT^2 lncRNA Inflammatory Response & Autoimmunity PCR Array PreAMP Primer Mix (QIAGEN, Germany). qRT-PCR was performed with RT^2 SYBR® Green qPCR MasterMix (QIAGEN, Germany) into the wells of RT^2 lncRNA PCR Array Human Inflammatory Response & Autoimmunity (QIAGEN, Germany) which

contains pre-dispensed gene-specific primer pairs for 84 lncRNA and 12 controls (for the complete list of genes see Appendix S1).

Thermal cycling conditions: holding stage at 95 °C for 10 min (enzyme activation), 40 cycles of each PCR step [(denaturation) 95 °C for 15 s and (annealing/extension) 60 °C for 1 min].

Statistical analysis

For statistical analysis, the cycle quantification (Cq) values were analyzed using the QIAGEN online software (https://www.qiagen.com/dataanalysiscenter). During statistical analysis, the Cq cut-off was set to 35 cycles in order to dismiss those lncRNA whose expression was too low, which could make their real-time quantification unreliable. Significant changes (p-value<0.05) that were at least two-fold up- or down-regulated, as compared to no pain group, were selected. With pre-amplification, two out of five reference genes *Ribosomal protein large P0* (*RPLP0*, NM_001002) and *RNA Component Of 7SK Nuclear Ribonucleoprotein* (*RN7SK*, NR_001445), were consistently detected at a high level, and their arithmetic mean was used as a reference for normalization [24].

Fold change/regulation is calculated using ΔΔCq method where ΔCq is calculated between the gene of interest and an average of reference genes, followed by ΔΔCq calculations [$\Delta Cq_{(pain)} - \Delta Cq_{(no\ pain)}$]. After, fold change is calculated using $2^{-\Delta\Delta Cq}$ formula according to Livak and Schmittgen [25]. Fold-change values >1 indicate an up-regulation, and the fold-regulation is equal to the fold change. Fold-change <1 indicate a down-regulation, and the fold regulation is the negative inverse of the fold-change. The p-values were calculated based on a Student's t-test of the replicate $2^{(-\Delta CT)}$ values for each gene in the no Pain group and Pain group. The data analysis was plotted with a volcano plot that combines a p-value (*y*-axis) with the fold change (*x*-axis) that allows identifying genes with both large and small expression changes statistically significant.

In silico target analysis of lncRNA interactions

The prediction analysis of lncRNA-miRNA interactions was performed, using starBase v. 3.0 (http://starbase.sysu.edu.cn/index.php) software a web-based tool that predicted miRNA-lncRNA interactions by scanning lncRNA sequences overlapping with CLIP-Seq peaks for potential microRNA targets [26, 27]. Moreover, to evaluate the biological process, in which the significant lncRNA are involved, a prediction analysis of lncRNA-RNA was run on starBase v. 3.0 and genes predicted were chosen for further functional enrichment analysis of biological processes, carried out using GeneOntology.org powered by PANTHER using Fisher's Exact test (FDR≤0.05). REVIGO (revigo.irb.hr) was used to reduce redundancy in the list of GO-terms, which were either close in GO hierarchy or related by inheritance [26–29].

Results

Demographic

Twenty patients with KOA (nine females and 11 males) were recruited. The two groups were not statistically different regarding gender, age, BMI, preoperative VAS, and radiological assessment of OA (the Kellgren Lawrence Scale), see Table 1.

Table 1: Demographics. Demographics of patients enrolled in the study. Data for age, Kellgren Lawrence scale, body mass index (BMI), and pre/postoperative VAS are presented as mean ± standard deviation (SD).

	No pain (n=10)	Pain (n=10)	p-Value
Gender	5 m; 5 f	6 m; 4 f	0.653[a]
Age, years	64.27 ± 8.88	61.82 ± 10.62	0.31[b]
Kellgren Lawrence (0–4)	3.7 ± 0.48	3.36 ± 0.69	0.281[b]
BMI, kg/m^2	25.5 ± 3.23	33.3 ± 6.52	0.36[b]
Preoperative VAS	8.0 ± 0.67	6.9 ± 2.16	0.22[b]
Postoperative VAS	0.18 ± 0.30	8.49 ± 1.38	>0.0001[b]

[a]p-Value obtained by the Chi-square test. [b]p-Value obtained by Student's t-test.

LncRNA expression profile in patients with post-operative pain

Three lncRNA that were significantly down-regulated in the Pain Group (Figure 1); *Myeloid Zinc Finger 1 Antisense RNA 1* (*MZF1-AS1*, ENST00000593642) showed down-regulation of −3.99 fold change (p-value: 0.038); also in the case of *Metastasis associated lung adenocarcinoma transcript 1*(*MALAT1*, NR_002819) was shown downregulation of −3.39 fold change (p-value: 0.044); *Patched 1 pseudogene* (*LOC100287846*, ENST00000528139) highlighted down-regulation of −6.99 fold change (p-value: 0.029).

Potential target genes of differentially expressed circulating lncRNA

In silico target prediction analyses were performed to highlight the potential miRNA, gene targets and the possible biological process regulated by the significant lncRNA. Using this open-source online software the number of miRNA targets for MALATI and MZF1-ASI was 357 and 68 respectively, highlighting that these two lncRNA are involved in the regulation of several pathways in which different miRNA takes action (e.g. pain, inflammation) (Appendix S2). LncRNA-RNA analysis showed 1,637 genes regulated by *MALAT1*, nine genes for *MZF1-AS1* (Appendix S2). The functional enrichment analysis of GO biological processes, showed 26 significant pathways (FDR≤0.05) regulated by *MALAT1*, which revealed involvement of this lncRNA in the inflammation cascade and his activity in response to stimuli (Figure 2). No significant enrichments were found for genes regulated by MZF1-AS1. Because

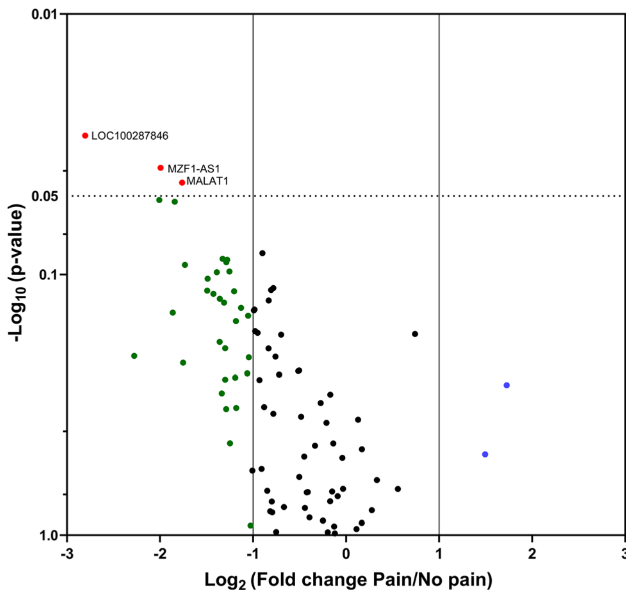

Figure 1: Volcano plot of lncRNA's fold changes comparing pain vs. no pain KOA patients. Statistical significance represented as $-\text{Log}_{10}$ of p-value vs. Log_2 of fold changes ratio between the two groups was shown on the *y*- and *x*-axes, respectively. Red dots indicate significantly downregulated lncRNA; green, black, and blue dots represent downregulated, unchanged, and upregulated lncRNA respectively. All cycle quantification (Cq) values were normalized to the arithmetic average of expression levels of *RPLP0* and *RN7SK*.

of the uncharacterized nature of *Patched 1* pseudogene *(LOC100287846)*, it was not possible to evaluate any eventual alignment with a sequence of specific miRNA or RNA targets.

Discussion

The current study is the first to assess preoperative circulating lncRNA as a serological preoperative signature for chronic postoperative pain after TKR. The findings indicate that preoperative downregulation of *Metastasis associated lung adenocarcinoma transcript 1 (MALAT1)*, *Myeloid Zinc Finger 1 Antisense RNA 1 (MZF1-AS1)*, and *Patched 1 pseudogene (LOC100287846) are* associated with chronic postoperative pain after TKR.

LncRNA modifications and pain in KOA

LncRNA have gained widespread attention due to their potential roles in many biological processes and pathological conditions [30]. Studies show how lncRNA are involved in several physiological and pathological processes acting in different pathways on several genes at transcriptional, post-transcriptional, and epigenetic levels [31, 32]. Moreover, LncRNA have been associated with responses to experimental pain [20, 22, 33–35] and has been suggested as a new target for future pain therapies [36, 37]. Previous studies have been looking deeply at the expression of different RNA and ncRNA in relation to the pathophysiology of OA [38, 39]. In 2018 Bratus-Neuenschwander A. and colleagues demonstrated dysregulation of several mRNA and ncRNA in synovial biopsies of KOA patients when comparing high and low pain patients [39]. The authors showed that two lncRNA, with unknown functions, were downregulated in patients that reported high pain score [39]. More recently, authors using a three-step computational method to construct a lncRNA interaction network in OA patients, identified eight lncRNA biomarkers in patients with osteoarthritis (OA) of the knee associated with mild pain and severe pain [38].

In this study, with an array qRT-PCR approach, three out of 84 circulating lncRNA were found preoperatively downregulated in the serum of KOA patients who developed chronic postoperative pain. *MALAT1* is a conserved and widely expressed lncRNA, described for the first time in lung cancer cells in relation to metastasis and invasiveness of this type of cancer [40]. A recent study found *MALAT1* to be down-regulated in spinal cord neurons of neuropathic pain rat model showing that down-regulation of *MALAT1* could result in neuropathic pain by increasing the excitability of spinal cord dorsal horn neurons, due to potential involvement in the regulation of the transmembrane flow of calcium ions, although the specific mechanism by *MALAT1* has not yet been reported [41].

MZF1-AS1 regulates the transcription of factor MZF1, a member of the SCAN-Zinc Finger (SCAN-ZF) transcription factor family [42]. MZF1 has been studied for cancer tumors and is involved in early 147 myeloid lineage differentiation and pro-inflammatory effector functions and in the etiology of different types of cancers such as hematopoietic, breasts and lungs [42, 43]. Unfortunately, little is known about *MZF1-AS1*, but its downregulation could cause upregulation of *MZF1*, due to its role as antisense. A study has shown the involvement of MZF1 transcription factor in the pathways of neuropathic pain in rat model of nerve injury [20]. In the present study, down-regulation of *MZF1-AS1* was found preoperatively in KOA patients who developed chronic postoperative pain suggesting that *MZF1-AS1* might be involved in processes that leads to altered regulation of ion channels causing the painful postoperative pain state.

LOC100287846 is an uncharacterized lncRNA, not present in literature with any evidence of its involvement in specific pathophysiological processes. In this study we

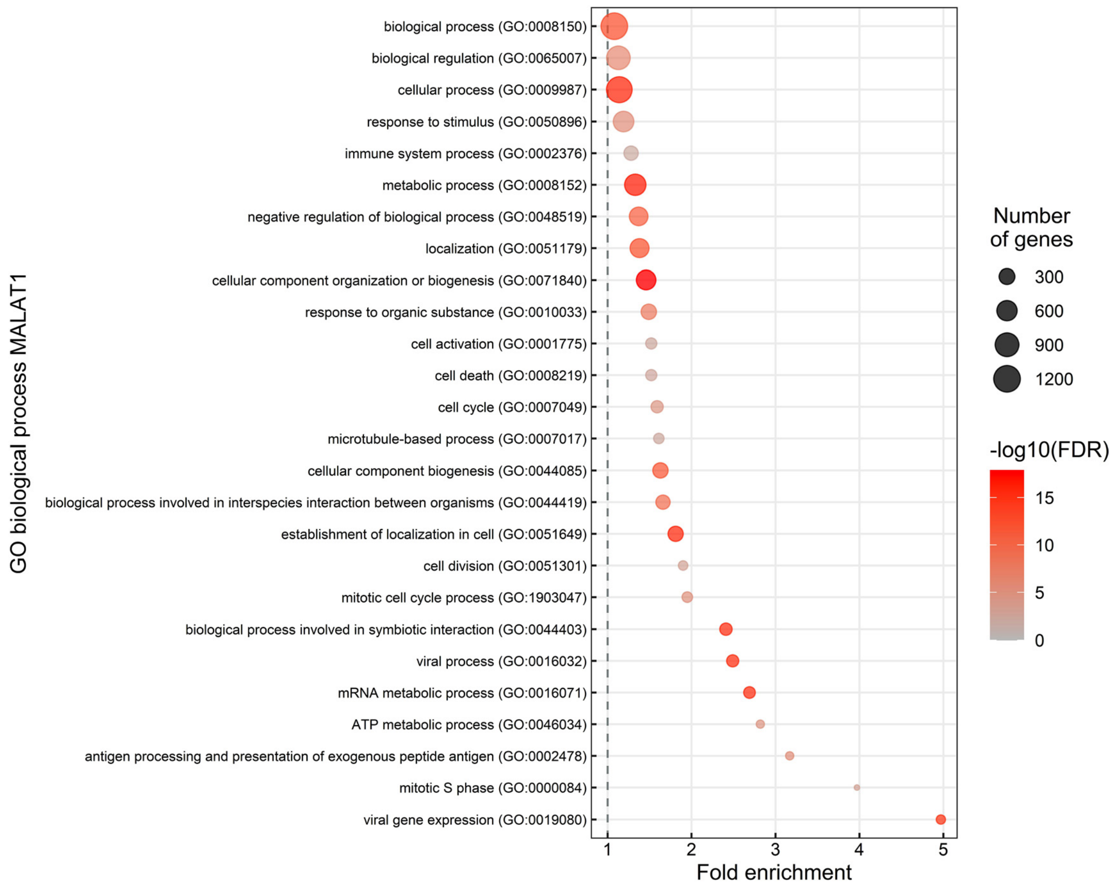

Figure 2: Bubble plot of enriched GO biological processes. Functional enrichment analysis showed 26 functionally enriched GO biological processes (FDR<0.05). Vertical dotted line indicates a fold enrichment of one.

demonstrate its potential association with post-operative pain one year after TKR. Further studies in different cohorts and pathological conditions will be needed in order to validate these findings.

In addition, the target miRNA prediction analysis showed a predicted interaction of *MZF1-AS1* and *MALAT1* with several miRNA, pointing to possible involvement of these two lncRNA in the regulatory pathways of these small non-coding molecules previously described to be involved in pain and inflammation processes [44–46].

In the long list of miRNA regulated by the action of the downregulated lncRNA, three like *miR-146a*, *145-5p*, *21a*, and *155* are some of the miRNA, which could be targeted by the two lncRNA.

They have been shown to be strongly involved in pain and inflammation pathways in previous studies [44, 47, 48]. *Hsa-miR-146a-5p* expression is induced by the action of pro-inflammatory mediators and its higher levels have been found in mouse model, in which this attenuates neuropathic pain behavior in the animal tested [49, 50]. Moreover, *hsa-miR-145-5p* has been shown to be expressed in the cerebral spinal fluid of patients with fibromyalgia correlated with pain intensity and fatigue [51]. *In silico* target prediction highlighted these two miRNAs as targets for MALAT1 and MZF1-AS1, confirming previous data reporting a synergic of lncRNAs, miR-146a-5p and 145-5p in process which regulates and promote pathways related with inflammation response and pain onset in OA [46, 52]. *Hsa-miR-155-5p* is expressed in the central nervous system [53] and has been shown to be involved in the regulation of inflammation diseases [54]. The expression of *hsa-miR-21a* has been shown to be associated with pain in patients with neuropathies [45]. This evidence gives the idea of how the preoperative action of lncRNA could possibly influence, at different levels, and regulate post-translational modification which could cause the development of low-grade systemic inflammation and pain after surgery in patients with KOA.

Limitations of the study

Patients assigned in this study to the "No Pain" group showed slight presence of postoperative pain in average. A possible pain intensity worsening was previously reported for pain intensity and quality of life (QoL) in KOA patients after primary total knee replacement and after revision surgery [3, 55], due to several factors which includes impaired sleep and anxiety [56, 57]. In this study the average of postoperative pain intensity was defined as absence of pain since the average value below one in VAS score reported [58].

In this study, the qRT-PCR was validated using, a standardized kit and several internal control assays (ACTB Actin beta; B2M Beta-2-microglobulin; RPLP0 Ribosomal protein large P0; RN7SK RNA, 7SK small nuclear; SNORA73A Small nucleolar RNA, H/ACA box 73A; HGDC Human Genomic DNA Contamination) and quality controls (reverse-transcription control RTC, genomic DNA control, positive PCR control PPC) but quantification of the extracted total RNA was not performed before the qRT-PCR, which could be pointed out as a limitation for the study. No replicates were run during qRT-PCR following manufacturer instructions. Due to the exploratory nature of the study correction for multiple testing, such as False Discovery Rate or Bonferroni, has not been run on the presented dataset.

Cellular derivation of circulating lncRNA is still unclear pointing out a difficult identification of cells capable of secreting and receiving circulating free and exosomal lncRNA [59–61], which makes it impossible to determine the tissue origin as well as the recipient cells of the presently observed dysregulated lncRNA.

Conclusions

This is the first study to demonstrate an association between the downregulation of preoperative lncRNA *Metastasis associated lung adenocarcinoma transcript 1 (MALAT1)*, *Myeloid Zinc Finger 1 Antisense RNA 1 (MZF1-AS1)* and *Patched 1* pseudogene *(LOC100287846)*, and chronic postoperative pain after TKR. This exploratory study gives the first insight into preoperative circulating lncRNA dysregulation and indicates their potential as biomarkers for postoperative pain conditions. Larger cohort studies are needed to confirm the value of these markers in clinical practice.

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Conflict of interest: Authors state no conflict of interest.
Informed consent: Informed consent has been obtained from all individuals included in this study.
Ethical approval: The research was performed in accordance with the tenets of the Helsinki Declaration, and has been approved by The North Denmark Region Committee on Health Research Ethics (N-20120015).

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