Eur J Vasc Endovasc Surg **26**, 32–38 (2003)
doi:10.1053/ejvs.2002.1907, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

Echolucency of Carotid Plaques Correlates with Plaque Cellularity

I. Gonçalves*[1,2,4], J. Moses[3], L. M. Pedro[4], N. Dias[4], J. Fernandes e Fernandes[4] J. Nilsson[1] and M. P. S. Ares[1]

[1]*Department of Medicine, Lund University, Wallenberg-laboratory, Malmö University Hospital, SE-20502 Malmö, Sweden,* [2]*Department of Cardiology, Malmö University Hospital, SE-20502 Malmö, Sweden,* [3]*Wallenberg Laboratory for Cardiovascular Research, Matrix and Proteoglycan Group, Sahlgrenska Academy at Gothenburg University, SE-41345 Gothenburg, Sweden and* [4]*Instituto Cardiovascular de Lisboa, R. Tomás da Fonseca, Torres de Lisboa, Torre F, 2°, 1600-209 Lisbon, Portugal*

Objective: *to analyse the relationship between carotid plaque echolucency and cellularity.*
Methods: *carotid plaques (14 symptomatic and 16 asymptomatic) were snap frozen after endarterectomy and defined on the basis of their grey-scale-median (GSM), obtained from pre-operative high-definition ultrasonography, as either echolucent (<32) or echogenic (≥32). DNA and total soluble protein were determined to assess cellularity.*
Results: *after correcting for wet weight, symptomatic plaques had significantly more DNA (0.400 ± 0.07 vs 0.335 ± 0.07 mg/g; p = 0.03) and soluble protein (34.1 ± 6.6 vs 29.7 ± 3.4 mg/g; p = 0.03) than asymptomatic plaques. Predominantly echolucent (Grey-Weale classification) plaques had more DNA (0.404 ± 0.06 vs 0.332 ± 0.08 mg/g; p = 0.03) than echogenic plaques. Plaques with GSM < 32 also had more DNA (0.386 ± 0.08 vs 0.319 ± 0.06 mg/g; p = 0.04) and soluble protein (34.7 ± 7.3 vs 29.6 ± 4.2 mg/g; p = 0.03) than those with GSM ≥ 32. Inverse relations were found between GSM and plaque DNA (r = −0.47; p = 0.02) and soluble protein (r = −0.45; p = 0.02) as well as between age and DNA (r = −0.39; p = 0.04) and soluble protein (r = −0.50; p = 0.003).*
Conclusions: *echolucency of carotid plaques as assessed by ultrasonography reflects plaque cellularity. This observation support the notion that ultrasonography can be used to identify high-risk plaques and evaluate effect of interventions on plaque structure.*

Key Words: Atherosclerosis; Carotid artery stenosis; Aging; Ultrasound; Echostructure; Cellularity.

Introduction

Embolization from ruptured atherosclerotic plaques is a major cause of ischemic stroke.[1] Vulnerable plaques are characterized by a thin fibrous cap covering areas of necrosis, extracellular lipid deposits and inflammatory infiltrates.[2–4] Several studies suggest that B-mode ultrasonography may be used not only to assess the severity of carotid stenosis[5–7] but also to identify vulnerable plaques.[4,8,9] Carotid plaques causing cerebrovascular symptoms and infarction have been subjectively characterized as echolucent.[4,9–14] The recent application of B-mode image grey scale intensity analysis using digital image processing has allowed the objective measurement of plaque echolucency.[15–18] Plaques with a low grey scale value are more common among symptomatic patients, correlate with a higher prevalence of cerebral infarction on computed tomography and predict increased risk for future stroke.[16,19,20] Histological analysis of carotid plaques removed at surgery have shown that plaques with a low grey scale value have more lipid deposition and haemorrhage. By contrast, echo-rich plaques contain more fibrous tissue.[15,17,21] Moreover, presence of major cardiovascular risk factors, such as hypertriglyceridemia, low HDL cholesterol and increased levels of circulating inflammatory markers, are also associated with echolucent plaques.[22] These observations suggest that analysis of carotid plaque grey scale intensity by B-mode ultrasonography can be used to assess plaque structure. This may help to identify high risk patients requiring pharmacological or surgical intervention, and be used to monitor the effect of non-invasive interventions and pharmacotherapy. The present study was designed to investigate if plaque cellularity, as assessed by DNA and soluble protein content, is related to echostructure.

* Please address all correspondence to: I. Gonçalves, University of Lund, Wallenberg-laboratory, University Hospital MAS, SE-20502 Malmö, Sweden. Tel.: +46-40-337659; fax: +46-40-332550; email: isabel.goncalves@medforsk.mas.lu.se

1078–5884/03/010032 + 07 $35.00/0

Material and Methods

Thirty plaques from 27 patients (21 males) with mean (SD) age of 67 (±8.5) years were immediately snap frozen after removal *en bloc* at carotid endarterectomy (CEA). Three patients had bilateral CEA. In one both, in another neither was symptomatic, and in the third only one plaque was symptomatic. Other causes for symptoms were excluded by complete neurologic and cardiologic evaluations, including CT-scan and echocardiography. Fourteen plaques were associated with ipsilateral hemispheric symptoms and corresponded to internal carotid artery stenosis greater than 70% (ECST).[23] Plaques removed from asymptomatic patients represented an internal carotid artery stenosis greater than 80% (ECST).[23] The study had the approval of the local research ethics committee. Informed consent was taken from each patient. Cardiovascular risk factors such as hypertension (systolic blood pressure >140 mmHg), diabetes, coronary artery disease, tobacco use (previous or current) were recorded. Total cholesterol, HDL (high density lipoprotein) cholesterol, LDL (low density lipoprotein) cholesterol and triglycerides were measured. Carotid high-definition ultrasonography (ATL-HDI3000, 7–10 MHz probe, 60 dB dynamic range and post-processing linear maps) of 27 plaques was performed preoperatively in blinded fashion by one observer. Plaques with an acoustic shadow, whose morphology could not be fully assessed were excluded. The severity of carotid stenosis was assessed by duplex Doppler imaging by the same observer (intra-observer variability $r = 0.89$) using the ECST criteria[23] and plaque cross-sectional area reduction. Images were digitalized, computer-standardized (Adobe Photoshop 3.0®) and the grey-scale-median (GSM) determined as previously described.[18,19] Plaques were classified according to Gray-Weale:[24] type I, homogenous echolucent; type II, heterogenous predominantly echolucent; type III, heterogenous predominantly echogenic; type IV, homogenous echogenic. Due to the small number of plaques types I and II (predominantly echolucent) and types III and IV (predominantly echogenic) were combined. The plaques were further classified according to GSM (<32 vs ≥32) as in previous studies this had provided the optimal discrimination between symptomatic and asymptomatic plaques.[19]

Frozen plaques were weighed and homogenized (5 ml 50 mmol/l tris HCl (pH 7.5), 0.25 mol/l sucrose, 2 mmol/l TCEP HCl (tris(2-carboxyethyl)phosphine), 50 mmol/l NaF, 1 mmol/l Na-orthovanadate, 10 mmol/l Na-glycerophosphate, 5 mmol/l Na-pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, protease inhibitor cocktail (Roche Complete™, EDTA-free), 1 mmol/l benzamidine, and 10 mmol/l PMSF) using a low temperature motorised (1600 rpm) Teflon pestle and a pre-cooled (−80 °C) Teflon chamber with four pre-cooled (−80 °C) agate grinding balls, at 3000 rpm (20 s pulses with cooling to −80 °C between pulses), using a Mikro-Dismembrator S (B. Braun Biotech International). Homogenate DNA content was measured using Pico-Green[26] ds DNA Quantization Kit (Molecular Probes) after proteinase K-treatment (Sigma) in 20 mmol/l Tris, pH 7.8, 0.07% SDS, 0.2% Triton X-100. The quantification was done according to the manufacturer's instructions in a 96-well fluorescence plate reader. To avoid background fluorescence, samples without PicoGreen probe were included in parallel readings. The instrument was pre-calibrated using reagent blanks with and without the addition of PicoGreen probe. Measurement of DNA was taken as a direct assesment of cellularity within the plaque tissue. Total soluble protein was determined according to the method of Lowry.[27] Results were corrected for wet weight and a $p < 0.05$ was considered significant. Values are presented as mean ± standard deviation. Chi-squared analyses or Fisher's exact test analyses were made to investigate associations with dichotomous variables. Two-group comparisons were performed using the unpaired Student's t test. Fisher's r to z test was used for the correlation analyses. Statistical analysis was performed using StatView for Windows, version 5.0.1 (SAS Institute Inc., Cary, CA, U.S.A.).

Results

There were no statistically significant differences between symptomatic and asymptomatic patients, except for plasma triglycerides (Table 1), which were higher in symptomatic patients than in asymptomatic ones. Ultrasound data for 3 samples were accidentally not saved (Table 2). Symptomatic plaques had higher DNA (Fig. 1A) and soluble protein (Fig. 1B) contents. Predominantly echolucent plaques had higher DNA (Fig. 2A) but not soluble protein (Fig. 2B), contents than predominantly echogenic plaques. Plaques with GSM < 32 had higher DNA (Fig. 3A), and soluble protein (Fig. 3B), contents than plaques with GSM ≥ 32. There was an inverse correlation between patients age, DNA (Fig. 4A) and soluble protein (Fig. 4B) contents. There was a positive correlation between DNA and total protein ($r = 0.74$; $p < 0.0001$) (Fig. 5). There was an inverse correlation between GSM and both DNA (Fig. 6A) and soluble protein (Fig. 6B).

Table 1. Cardiovascular risk factors in symptomatic and asymptomatic patients.

Risk factor	Symptomatic	Asymptomatic	*p*
Hypertension	8 pts	10 pts	NS
Diabetes	2 pts	4 pts	NS
Coronary artery disease	7 pts	10 pts	NS
Tobacco past use	4 pts	2 pts	NS
Tobacco current use	2 pts	5 pts	NS
Serum lipids (mg/dL)			
Total cholesterol	226.5 ± 35.3	219.1 ± 53.1	NS
HDL cholesterol	42.9 ± 10.7	51.6 ± 15.4	NS
LDL cholesterol	150.2 ± 33.1	144.4 ± 35.8	NS
Triglycerides	171.2 ± 101.2	102.9 ± 46.3	0.03

Pts, patients; NS, not significant.

Table 2. Number of plaques in each group, for each variable.

Plaques	*n*
Associated with symptoms vs not associated with symptoms	
DNA	14 vs 16
Soluble protein	12 vs 16
Predominantly echolucent vs predominantly echogenic	
DNA	12 vs 12
Soluble protein	14 vs 13
With GSM < 32 vs with GSM ≥ 32	
DNA	10 vs 13
Soluble protein	10 vs 17

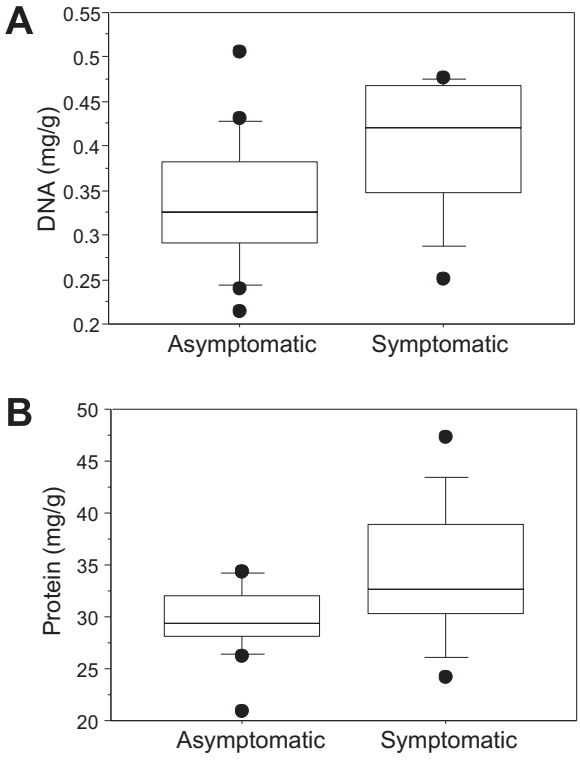

Fig. 1. (A) Carotid plaques associated with ipsilateral neurological symptoms have higher amounts of DNA than those from asymptomatic patients (0.400 ± 0.07 vs 0.335 ± 0.07 mg/g wet weight plaque; $p = 0.03$). (B) Plaques associated with symptoms have higher amounts of soluble proteins than those from asymptomatic patients (34.1 ± 6.6 vs 29.7 ± 3.4 mg/g plaque wet weight; $p = 0.03$).

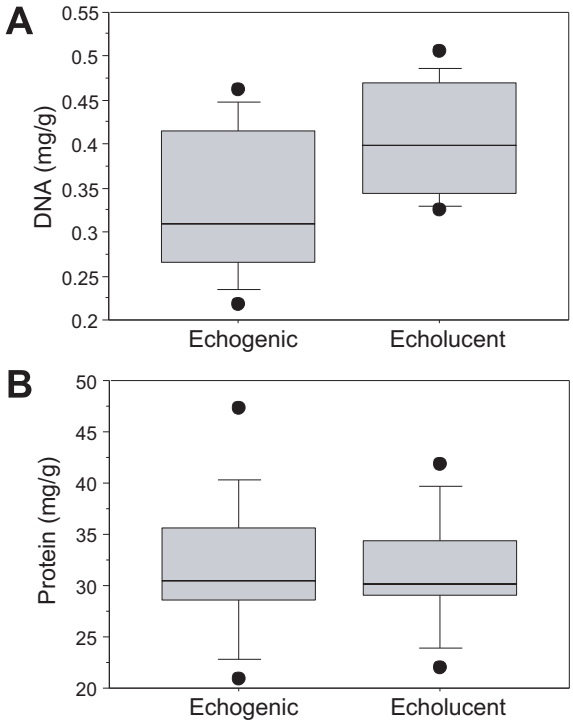

Fig. 2. Comparison of plaques classified by non-standardized high-definition carotid ultrasonography into predominantly echolucent (types I and II of Gray-Wheale classification) and predominantly echogenic (types III and IV of Gray-Wheale classification) plaques. (A) Predominantly echolucent plaques have more DNA than predominantly echogenic ones (0.404 ± 0.06 vs 0.332 ± 0.08 mg/g wet weight plaque; $p = 0.03$). (B) On the other hand, no statistically significant difference was found between predominantly echolucent and predominantly echogenic plaques concerning the amount of soluble protein (31.2 ± 5.3 vs 31.7 ± 6.8 mg/g plaque wet weight).

Discussion

Most studies on the composition of atherosclerotic plaques and its assessment by ultrasound techniques have relied on histology. Analysis of total levels of plaque components is, however, more appropriately done by biochemical assays of whole plaque homogenates, corresponding to total plaque composition.

Non-invasive analysis of plaque structure, for example by spiral CT,[28–30] MRI[31–35] and ultrasound, may help identify high risk patients and monitor the effects of pharmacological interventions. Standardized computer-assisted high-definition ultrasonography can overcome the error and bias inherent in obtaining data from different machines, centres and individual sonographers. It is accepted that echolucent plaques contain more lipid[17,21,22] and, specifically, that there is an inverse association between GSM and lipid content.[17,21,22] The present study extends these findings by demonstrating that echolucent plaques (GSM < 32) are more cellular than echorich plaques. The

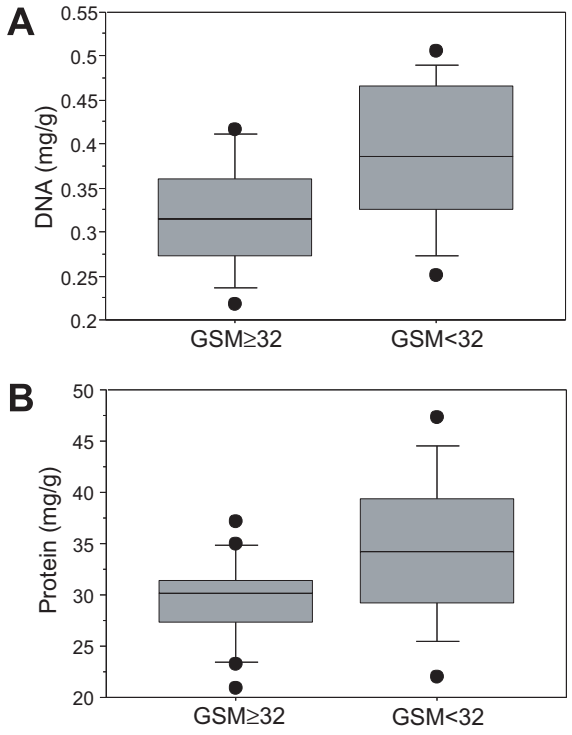

Fig. 3. Comparison of plaques classified by standardized high-definition carotid ultrasonography. The gray-scale-median (GSM) was obtained by standardized computer assisted image analysis. (A) Plaques with GSM < 32 have higher DNA content than plaques with GSM ≥ 32 (0.386 ± 0.09 vs 0.319 ± 0.06 mg/g plaque wet weight; $p = 0.04$). (B) Additionally, plaques with GSM <32 also have higher levels of soluble protein than plaques with GSM ≥ 32 (34.7 ± 7.3 vs 29.6 ± 4.2 mg/g plaque wet weight; $p = 0.04$).

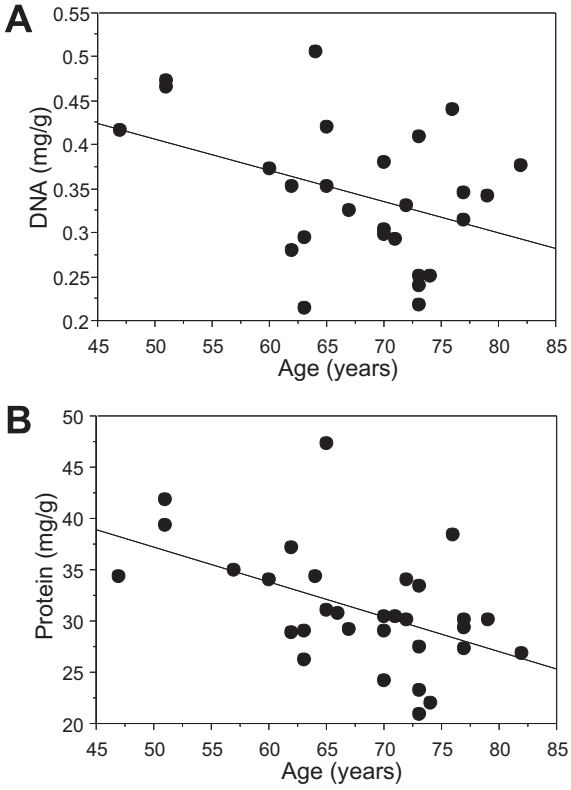

Fig. 4. Relation between cellularity and age. (A) Inverse correlation between DNA (mg/g plaque wet weight) content in the atherosclerotic carotid plaques and the age of the patients (years) ($r = -0.39$; $p = 0.04$). (B) Inverse correlation between the soluble protein content in the carotid plaques (mg/g plaque wet weight) and the age of the patients (years) ($r = -0.50$; $p = 0.003$).

determination of DNA and soluble protein has both advantages and disadvantages as compared to histological analysis.[36–40] Although DNA is cell specific there is a theoretical risk of contamination by microorganisms found in plaque.[41–44] Most components of the extracellular fibrous matrix, such as collagen and elastin, are insoluble and were, therefore, not measured in the current study. Small proteins associated with extracellular matrix fibers, extracellular enzymes and lipoproteins represent part of the soluble proteins in plaques, but cellular proteins are likely to constitute the major part of the plaque soluble proteins. This notion is also supported by the observation of a strong association between plaque DNA and soluble protein. We have not found other studies reporting the content of soluble proteins in atherosclerotic lesions. However, soluble protein in tumor tissue has been shown to be a good index of cellularity.[45]

The major disadvantage with using biochemical techniques to determine plaque cell content is that they do not allow identification of different cell types. Atherosclerotic plaques contain endothelial cells, smooth muscle cells and inflammatory cells, primarily

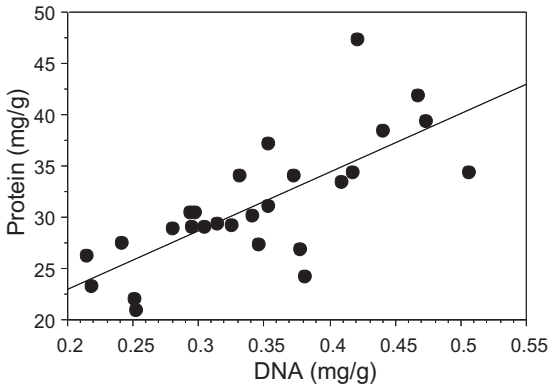

Fig. 5. Positive correlation between the amount of soluble protein (mg/g plaque wet weight) and the amount DNA (mg/g plaque wet weight) in the carotid plaques ($r = 0.74$; $p < 0.0001$).

macrophages and T cells.[46–51] Endothelial cells constitute only a minor fraction of the cells in a plaque and are not likely to differ significantly between echolucent and echorich plaques. Smooth muscle cells are located in the fibrous part of the plaque, where they usually are sparsely distributed. Fibrous, lipid-poor plaques

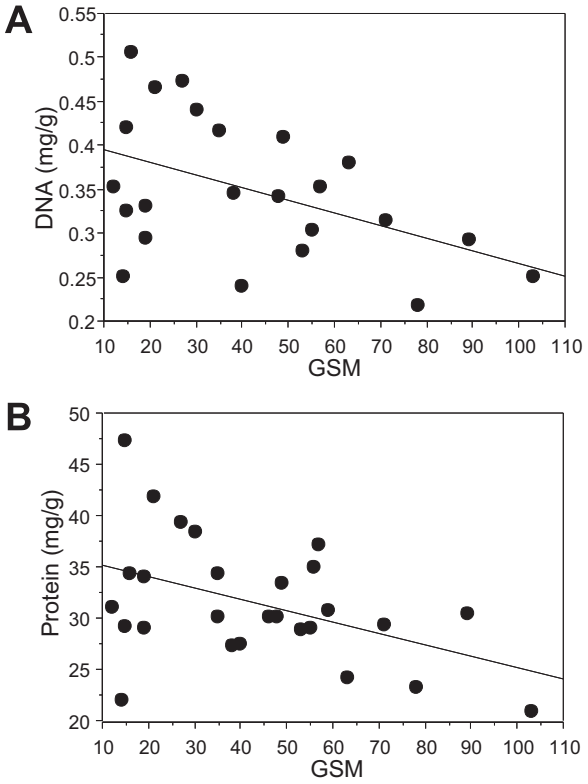

Fig. 6. (A) Inverse correlation between plaque DNA (mg/g plaque wet weight) and the gray-scale-median (GSM) obtained by high-definition ultrasonography with computer-assisted image analysis of the carotid plaques ($r = -0.47$; $p = 0.02$). (B) Inverse correlation between soluble protein (mg/g plaque wet weight) and the gray-scale-median (GSM) ($r = -0.45$; $p = 0.02$).

contain mostly smooth muscle cells, and cell density in fibrous plaques has been shown to be lower than in lipid-rich lesions.[52] Macrophages and T cells are more common in lipid-rich plaques, where they are located in cell-dense infiltrates.[2,49,50,53] Hypercholesterolemia has been reported to increase the rate of protein synthesis in rabbit atherosclerotic lesions.[54] Increased levels of soluble protein may therefore reflect either an increased number of cells or an increased activity of cells. In the present study, DNA and soluble protein levels were found to be closely correlated, suggesting that soluble protein is mainly a measure of cellularity. This notion is also supported by the observation that echorich plaques contain lower levels of both DNA and soluble protein. Atherosclerotic lesions become more fibrotic with increasing age.[55] Accordingly, we found an inverse relation between age and plaque cell content, with similar results for DNA and soluble protein. This suggests that plaques from younger patients, in whom lesions are known to progress more frequently,[11] might be more vulnerable, possibly demanding closer follow-up or even earlier intervention.

In summary, this study highlights the value of standardized B-mode ultrasonography and demonstrates that there is a significant association between the grey scale intensity as assessed by B-mode ultrasonography and the plaque cell content. It has previously been shown that there is an association between echolucency and plaque lipids.[17,21,22] It is possible that the association between echolucency and plaque cellularity observed in the present study primarily reflects differences in plaque lipid content. These observations add further support to the notion that standardized B-mode ultrasonography could be a useful instrument to identify patients with vulnerable, high-risk plaques and to monitor the effect of pharmacological interventions.

Acknowledgements

This study was supported by grants from The Swedish Research Council (grant number 8311), The Swedish Heart and Lung Foundation, The Swedish Medical Society, Malmö University Hospital funds, Crafoord Foundation, Ernhold Lundström Foundation, The Royal Physiographic Society, The Wallenberg Foundation and Lars Hierta Foundation.

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Accepted 3 February 2003