Two-tier approach for the detection of alpha-galactosidase A deficiency in a predominantly female haemodialysis population

Wim Terryn1,2,*, Bruce Poppe3,*, Birgitte Wuyts4, Kathleen Claes3, Bart Maes5,6, Dierik Verbeelen5,7, Raymond Vanholder1, Koen De Boeck8, Norbert Lameire1, Anne De Paepe3 and Gert De Schoenmakers1,6,*

1Department of Nephrology, Ghent University Hospital, Ghent, 2Department of Nephrology and Internal Medicine, Regional Hospital Jan Yperman, Ypres, Belgium, 3Centre for Medical Genetics, 4Department of Clinical Biology, Ghent University Hospital, Ghent, 5Representative of NBVN (Nederlandstalige Belgische Vereniging voor Nefrologie), 6Department of Nephrology and Internal Medicine, Heilig Hart Hospital, Roeselare, 7Department of Nephrology, University Hospital Brussels, Brussels and 8Department of Nephrology, ZNA Middelheim, Antwerp, Belgium

Abstract

Introduction. Fabry’s disease (AFD) is an X-linked lysosomal storage disease, resulting from a deficiency in alpha-galactosidase A (AGALA). Untreated, this leads to precocious failure of vital organ function and death. As enzyme replacement therapy is available, it is of vital importance that affected individuals can be traced.

Materials and methods. We set up a screening in the Flemish haemodialysis population using a two-tier approach. The first tier was a determination of alpha-galactosidase A activity using a dried blood spot on filter paper, in the second tier, patients with the lowest alpha-galactosidase levels were further subjected to mutation analysis of the GLA gene.

Results. 1284 patients (1047 women, 237 men) were evaluated for inclusion, eliminating patients with definite renal diagnoses. Total 922 patients (71.8 %) were screened (742 women, 180 men). Fifty seven patients were subjected to further genetic analysis. Three GLA mutation carriers were identified: two apparently nonrelated female patients carry the missense mutation p.Ala143Thr (c.427G > A), a missense mutation p.Trp236Arg (c.706T > C) was identified in a man. While the male patient had been clinically diagnosed with AFD, the female patients had remained unrecognized. Additional family based screening resulted in the identification of nine mutation carriers (four males and five females).

Discussion. We demonstrated that the prevalence of GLA mutation carriers in our haemodialysis population is 0.3%. Our results show that the proposed approach accurately detects AFD patients. We conclude that screening for AFD in high risk populations is a cost-effective, technically feasible and clinically valuable objective.

Keywords: alpha galactosidase; Fabry’s disease; female; haemodialysis; screening

Introduction

Fabry disease (AFD) is an X-linked sphingolipidosis resulting from a quantitative or functional deficiency in alpha-galactosidase A (AGALA) [1,2]. Its reported incidence ranges from 1/40 000 to 1/117 000 live births [3,4]. This enzyme defect leads to multiple organ dysfunction; in childhood, the invalidating neuropathic pain predominates. As from the second decade of life, renal, cardiac and neurological symptoms become apparent [4]. Due to its mode of inheritance, all affected males develop clinical symptoms; as a result of skewed lyonisation female carriers can develop the entire spectrum from asymptomatic carriers to classical AFD [5–7].

Several reports indicate that AFD is an underdiagnosed disease entity. This can be attributed to various factors. First, clinical awareness might be low in atypical presentations. In addition, an overrepresentation of female relatives can obscure signs and symptoms in a condition with X-linked inheritance. Finally, the identification of genetic aberrations with a reduced penetration or variant phenotype is anticipated from increasingly sensitive screening analyses.

With the introduction of a safe and efficacious treatment for AFD, the implementation of screening programs in high risk populations seems a clinically

Correspondence to: Gert De Schoenmaker, MD, Department of Nephrology and Internal Medicine, Heilig Hartziekenhuis Roeselare, B-8800 Roeselare, Belgium. Email: gdeschoenmaker@hhr.be

*These authors contributed equally to this work.
relevant objective. Identification of index cases in this setting often leads to detection of affected family members and opens possibilities for early enzyme replacement therapy.

We set up a community-wide screening program in Flemish haemodialysis centres, based on a two-tier approach: initial screening for AGALA deficiency using the dried blood spot technique (DBFP) [22], followed by standard genetic GLA gene mutation analysis of the high-risk persons identified in the first part of the study.

Materials and methods

Patient selection

The NBVN (Nederlandstalige Belgische Vereniging voor Nefrologie; a nephrology society grouping all 27 Flemish dialysis centres) database was used for patient selection. This database contains regularly updated diagnostic data on all Flemish haemodialysis patients (n = 2828 at the moment of inclusion), and also specifies the strength of evidence for these diagnostic data. Haemodialysis patients without biopsy-proven renal diagnosis or without evident reason for dialysis requirement (e.g. polycystic kidney disease, bilateral nephrectomy) were considered. Type 2 diabetes was not an exclusion criterion. Both genders were included, however with different age criteria: women older than 18 years (no upper age limit) and men between 18 and 60 years (at the moment of study) were included. In this patient group, one male patient was known to have AFD.

Determination of AGALA using the technique of dried blood spot sampled in filter paper

The screening test was based on a technique of a dried blood spot sampled in filter paper (DBFP) as described by Chamoles et al. [22]. In brief, a standardized disc was punched out of the filter paper and consequently incubated at physiologic pH and at 37°C with 4-methyl-umbelliferyl-α-galactopyranoside as substrate. Enzyme activity renders the enzyme product 4-methyl umbelliferone (4-MU), a fluorescent molecule. The fluorescence (excitation, 365 nm; emission, 450 nm) was measured on a Thermo Life Science fluorometer (Thermo Electron Corporation, Waltham, MA-USA). The fluorescence readings were corrected for blanks, and the results were compared with the fluorescence from a 4-methylumbelliferone calibrator. Enzymatic activities were expressed as micromoles of substrate hydrolysed per litre of blood per hour. To validate this technique in our laboratory setting, we performed an analysis of 50 patient samples (non-nephrology, non-ICU, non-haematology, non paediatric). Results were compared with literature data available. Each DBFP test for AGALA was validated by measurement of β-galactosidase; if no β-galactosidase activity was detectable in the DBFP test, the sample was rejected.

Determination of AGALA in white blood cells

Where appropriate, determination of AGALA levels in white blood cells were determined using the technique previously described by Desnick et al. [23].

Cut-off value for DNA mutation analysis

Because of the different distribution in the AGALA test results (see later) no cut-off value was used to select which patients could enter the second level of our two-tier screening analysis (mutation analysis). Instead, we performed mutation analysis of the GLA gene in patients within the lowest sixth percentile. This value was arbitrarily chosen, taking into consideration the feasibility and costs of the second tier of our protocol.

DNA mutation analysis

Genomic DNA was extracted from EDTA blood of the patients by standard protocols (PUREGENE DNA purification kit, Gentra) according to manufacturer’s instructions. Mutation analysis was performed by PCR amplification followed by direct sequencing of the seven exons and flanking intronic sequences of GLA (Genbank: X14448.1-genomic). Primers used were modified from Eng et al. [24].

Informed consent and ethics

The study protocol was approved by the Ethics committee of the Ghent University Hospital and all patients gave written or oral witnessed consent to participation. The study protocol is in accordance with the Declaration of Helsinki.

Results

Patient selection

All but one haemodialysis centre participated in the screening study (n = 26). From the 2828 patients in the database, 1284 (1047 women, 237 men) were selected after application of the inclusion criteria. Eventually, after informed consent, 922 patients (71.8%) were screened (742 women, 180 men).

AGALA levels in the control population

DBFP analysis of the 50 controls yielded a mean AGALA of 3.17 ± 1.6 μmol/l/h (2.5 and 97.5 percentile were 1.62 and 8.39 μmol/l/h, respectively). This is in accordance with literature data showing mean AGALA in healthy males of 2.93 ± 1.7 μmol/l/h (2.5 and 97.5 percentile were 1.37 and 7.66 μmol/l/h, respectively).

AGALA levels in the screening population

DBFP analysis of the 922 haemodialysis patients yielded a mean AGALA of 1.57 ± 1.5 μmol/l/h (2.5 and 97.5 percentile were 0.0001 and 5.07 μmol/l/h, respectively). This was significantly lower than the activity in the control group. This difference in AGALA was confirmed by performing a determination of AGALA on white blood cells: in 31 haemodialysis patients the mean enzyme activity was 3.67 ± 1.2 μmol/l/10⁹ WBC (2.5 and 97.5 percentile were 1.37 and 5.77 μmol/l/10⁹ WBC, respectively); in 50 healthy individuals the mean enzyme activity was...
5.43 ± 2.2 μmol/l/10^9 WBC (2.5 and 97.5 percentile were 2.07 and 9.91 μmol/l/10^9 WBC, respectively) (Figure 1). Neither gender nor presence of β-galactosidase had any influence on this difference.

**DNA mutation analysis**

Fifty-seven patients were subjected to further genetic analysis. Two non-related female patients were found to carry the p.Ala143Thr (c.427G>A) missense mutation. Another missense mutation p.Trp236Arg (c.706T>C) was found in a male patient who was already diagnosed with AFD before the start of the study. Both missense mutations are pathogenic and have been reported previously in AFD patients [25,26].

**Clinical correlation and family screening**

Index patient 1 (elaborate pedigree is shown in Figure 2) was 74 years old on her first admission to the hospital participating in the screening program. She was known to have chronic hypertension and had a creatinine of 87 μmol/l 1 year before admission. On admission, she presented with cardiac angina. At that time, she had nephrotic range proteinuria (11 g/l) and renal insufficiency (creatinine 407 μmol/l). As she refused kidney biopsy, the renal insufficiency was attributed to nephroangiosclerosis and the frequent use of non-steroidal anti-inflammatory drugs. The further work-up revealed she had left ventricular hypertrophy with a septum thickness of 15 mm on the first and 17 mm on the second echocardiography. Also, during hospitalization she developed a homonymous quadrantal anopsia with CT imaging suggestive of an ischaemic lesion in the left occipital region. The patient has since been in dialysis (for >5 years now) without major problems. When elaborating the patient’s history further, she revealed that after her first pregnancy (still birth), she developed invalidating pain in her lower limbs which, although not completely fitting the description of acroparesthesia, may be related to her underlying condition. The only additional clinical information on this family that is available is the phenotype of the 41-year-old nephew (Figure 2). This patient was found to have the mutation, but did not have any clinical sign of AFD: no symptoms, no cardiac and brain MRI abnormalities, normal renal biopsy, no proteinuria, normal renal function, normal echocardiography).

Index patient 2 (elaborate pedigree is shown in Figure 3) presented with cardiac angina at the age of 64. A coronarography showed three-vessel disease. At that time, renal function was normal, there was no proteinuria. A coronary arterial bypass grafting (CABG) was performed. Seventeen years later, she was referred again for cardiac angina. Coronarography showed recurrence of coronary ischaemia and aortic valve sclerosis. At that time, creatinine had risen to 168 μmol/l, there was no proteinuria. Imaging revealed...
she had an atrophic left kidney, and a right renal artery stenosis. The latter was dilated, and she underwent another CABG. The renal insufficiency was attributed to nephroangiosclerosis and ischaemic nephropathy. The renal insufficiency deteriorated, and the patient was started on chronic haemodialysis. Echocardiography performed on multiple occasions never showed left ventricular hypertrophy, nor were there signs of cerebral ischaemia. She died of cardiac arrest during dialysis. It is not clear whether the renal failure in this patient can be attributed to AFD; as the patient did not have proteinuria and taking into account the stenosis of the right renal artery and the atrophic left kidney, an alternative aetiology (renal ischaemia and vascular kidney disease) seems at least as likely. No additional clinical information is available on the other affected family members.

The third index patient had already been diagnosed with Fabry disease before the screening project, and had classical AFD. Overall we screened 17 relatives of the two newly found index patients, which resulted in the identification of four male hemizygotes and five female heterozygotes (Figures 2 and 3). Clinical evaluation and therapeutic counselling is ongoing and will be reported elsewhere.

Discussion

A screening programme for a rare genetic disease is time-consuming and expensive. Therefore, some prerequisites are indispensable: confinement of the screening to a well-defined population subgroup with a higher risk for the presence of the disease, the availability of a cheap and sensitive test method and the possibility to offer therapeutic and genetic counselling after a diagnosis is made [27].

Given the incidence data reported in literature [3,4], the number of patients carrying a mutation in the GLA gene in Flanders ranges from 50 to 150. In end-stage renal disease (haemodialysis subgroup), however, a much higher prevalence has been reported, ranging from 1.17% in Japan (exclusively men) [12], 0.22% in the Netherlands (exclusively men) [11] to 0.16% and 0.20% in Austria and the Czech Republic (both genders) [13,32]. Hence, based on literature data at least four GLA mutation carriers should theoretically be present in the Flemish dialysis population (n = 2828).

In the current report, we evaluated the clinical utility and the biochemical and molecular efficacy of a two-tier approach for AFD screening in haemodialysis patients without a definite renal diagnosis. It should be pointed out that excluding patients with a biopsy-proven renal diagnosis, leaves the possibility that some of the renal pathologists reading the biopsies might have overlooked the possibility of AFD.

We took advantage of the well-documented registry of our dialysis society to eliminate patients with a biopsy-proven renal diagnosis from the screening programme. The principle of the two-tier approach in this study was to further narrow down the number of patients using the relatively sensitive DBFP technique,
and then to submit these selected patients to the golden standard of mutation analysis.

Both genders were included in the screening protocol. Taking into consideration the knowledge on the natural history of Fabry disease [28,29] at the time of the study design, an upper age limit of 60 years in men was adopted. Meanwhile, however, Nakao et al. [12] reported ‘renal variants’ in older dialysis patients without classical symptoms. Hence, retrospectively, it would have been better to include the older male patient population. In women however, no upper age limit was applied, as skewed lyonisation can cause one organ to be severely affected, whereas other organs can be relatively spared [5]. As a consequence, in the relatively old Flemish haemodialysis population (mean age 68.8 years), more women than men were subjected to our screening protocol. In male haemodialysis patients our study may be underpowered to obtain representative results.

We decided to include and even focus on women in our screening programme, keeping in mind the major limitations of the DBFP test in this patient group. Linthorst et al. [30] recently demonstrated that one-third of the female carriers are missed using this test method. Currently no cost-effective alternatives can be applied in daily practice. Whereas in men a low AGALA activity is diagnostic for Fabry disease, the only way to make the definite diagnosis in women is through mutation analysis. This is time-consuming and expensive, hence beyond the scope of a screening programme. Given the poorer sensitivity of our DBFP test in women, we were still able to detect two patients that would not have been detected by excluding them completely from the screening protocol.

The DBFP results in all haemodialysis patients tested were somewhat surprising, as the enzyme activity was significantly lower than the reference value in literature [13,22] and that obtained from our own reference population (these last two were not significantly different). This difference was again confirmed by sampling of AGALA enzyme activity in WBC, both in 50 control samples and 31 samples obtained from haemodialysis patients. This contrasts with literature data: in the Dutch screening study, previously frozen whole blood samples were examined using a similar fluorescence technique. Values obtained were more than 10-fold higher, however excitation and emission spectra were different and no reference values were given [11]. In the Japanese screening study [12], plasma activity was measured using the same fluorescence technique, with resulting values in the range comparable to that of healthy controls. However, no whole blood samples were examined from the screened population. Finally, in the Austrian nationwide screening [13], no data are available on the distribution of enzyme activity in comparison with that of a control population. Our study demonstrated a remarkable and significantly lower enzyme activity in whole blood samples taken from dialysis patients, than in those taken from controls or found in literature data.

We excluded interference of uraemic toxins with our fluorescence method by determining the enzyme activity in WBC, which rendered similar results. Blood samples were taken before dialysis start, before patients had received anticoagulation. Further research in this field is needed to determine which factor is responsible for this decreased enzyme activity in patients on dialysis.

In adopting a two-tier approach in the protocol we tried to reduce the costs of the screening program. The costs of a single blood spot analysis in our centre is 5 euro, hence the first tier of the screening protocol costed 4610 euro. The second tier (genetic analysis) is more costly (300 euro per genetic analysis), but the total number of patients to be examined was reduced by the first tier. The second tier of the screening protocol costed 17 100 euro. Hence, the total screening cost was 21710 euro. Had we ideally screened all male patients by a blood spot analysis and all female patients by a mutation analysis, the cost of our screening study would have amounted to $(5 \times 180) + (300 \times 742) = 223 500$ euro, resulting in more than a 10-fold increase in costs. Extrapolating this to all haemodialysis patients, this would have been even more accentuated. We considered our screening methods as the most cost-efficient, taking into account that according to Linthorst et al. we may have missed one third of the female patients and taking into account that we may have missed some older male patients and some patients with a biopsy-proven renal diagnosis.

Our results confirm the performance of the adopted methodology in detecting GLA mutations, even in atypical clinical settings. While the male patient carrying the p.Trp236Arg was known with AFD, the p.Ala143Thr, previously reported in later-onset patients (e.g. [31]) and showing a considerable amount of transient expression in lysosomes [26], was detected in female patients, unsuspected of having AFD. Therefore screening efforts are expected to result in the detection of GLA mutations in patients presenting with variable phenotypes, even—as we demonstrated—in those with an attenuated phenotype.

The issue should be raised how not to miss the diagnosis in the future in these patients presenting with a non-classical pattern. In retrospect, in the first index patient some findings might have triggered the diagnosis of AFD; renal failure, left ventricular hypertrophy, cerebral thrombosis, lower limb pain. However, in this setting, the working hypothesis of polyvascular disease was at least as acceptable, keeping in mind the respective incidences of these affections. The second index patient illustrates that AFD may easily be missed, and even be impossible to diagnose, without a screening program.

Clearly, in individual patients it is of pivotal importance to detect the disease as early as possible in its natural course. By then, some useful interventions can still be made to slow down, stabilize, or even reverse the end-organ damage. Given the low incidence of AFD, this will always remain problematical—even
more so in patients with attenuated phenotypes. Only well-established population and physician information campaigns can help to improve the awareness of AFD and its symptoms. One step further, it is more rewarding to screen high-risk groups (renal failure, hypertrophic cardiomyopathy, cerebrovascular disease). Steps should be undertaken to implement routine measurements of AGALA in these patients, supported by recommendations in national and international guidelines. As an illustration, in the Flemish ESRD patients, we are planning to perform a measurement of AGALA as a standard procedure when entering our registry. This could give us the opportunity to prospectively detect possible AFD carriers, which is even more useful than the transsectional study we performed in this report. Although finding an index patient in this late stage of disease is less advantageous for the patient him/herself, it is certainly useful to detect family members in earlier stages.

AFD leads to vital organ failure and early death if untreated in men [29]. In women, the phenotype is largely dependent on lyonisation and the entire clinical spectrum is possible [5]. Given the multi-system involvement of the disease in adulthood (central nervous system, heart, kidney), and the high costs of lifelong enzyme replacement therapy (ERT), we advocate the centralization of data on the natural history of the disease and on effect of treatment on different organ systems. The two-tier approach—suitable for the current setting—may in the future be substituted by a one-tier approach once high-throughput mutation analysis becomes readily available. In addition, a state-of-the art treatment should not only focus on ERT alone, but should be holistic in preventing disease progression by delivering basic support when needed. Further steps are taken to extend our present screening programme to transplanted patients and patients on peritoneal dialysis. It might also be considered to screen for AFD in patients with mild to moderate renal function impairment. The diagnosis of AFD in these patients might be even more vital as adequate enzyme replacement therapy and standard supportive measures might stabilize or slow down the progression to end-stage renal disease in this group.

To date the first index patient is not treated by enzyme replacement therapy. This decision was made by the treating physicians based on her age and moderate phenotype (apart from the renal and cardiac involvement). However, her prominent left ventricular hypertrophy might warrant enzyme treatment. The usefulness and effect of ERT on morbidity and mortality in a dialysis population over 80 have not been substantiated in a large series and opens the discussion on treatment options in this type of patient. As further observational studies will be needed to provide us with the answer to this question, we suggest in the meantime make the decision on ERT initiation based on the clinical patient status and the expected survival on dialysis, even without AFD.

As mentioned, the second index patient died. We have no further information on the treatment of affected family members.

_Acknowledgements._ This study was supported by a grant by Genzyme to the NBVN Registry. The authors wish to thank all participating NBVN centres for their efforts to participate in the screening program.

_Conflicts of interest statement._ None declared.

**References**


Received for publication: 8.1.07
Accepted in revised form: 11.7.07