Dyslipidemia, sense, antisense or nonsense?
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Citation for published version (APA):
Visser, M. E. (2011). Dyslipidemia, sense, antisense or nonsense?

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Characterization of non-obese diabetic patients with marked insulin resistance identifies a novel familial partial lipodystrophy-associated PPARγ mutation (Y151C)

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* These authors contributed equally
Abstract

Background
Familial partial lipodystrophy (FPLD) is a rare metabolic disorder with clinical features that may not be readily recognised. As FPLD patients require a specific therapeutic approach, early identification is warranted. In the present study we aimed to identify cases of FPLD among non-obese patients with type 2 diabetes mellitus and marked insulin resistance.

Methods
We searched the databases of three diabetic outpatient clinics for patients with marked insulin resistance, arbitrarily defined as the use of ≥100 U insulin/day, and BMI ≤27 kg/m². In all patients, metabolic variables and anthropomorphic measurements were evaluated and DNA was sequenced for mutations in the genes encoding lamin A/C (LMNA), peroxisome proliferator-activated receptor γ (PPARG) (PPARG) and cell death-inducing DFFA-like effector c (CIDEC).

Results
Out of 5,221 diabetic individuals, 24 patients fulfilled all criteria. Twelve patients were willing to participate, of whom five showed clinical features of lipodystrophy. In three of these patients the clinical diagnosis of FPLD was confirmed by the presence of mutations in LMNA or PPARG; one patient harboured a novel heterozygous mutation (Y151C) in PPARG. The Y151C mutant displayed impaired DNA-binding capacity and hence reduced transcriptional activity compared with wild-type PPARγ. Dominant-negative activity was absent.

Conclusion
The combination of BMI ≤27 kg/m² and the use of >100 U insulin/day increases the chance of identifying lipodystrophy. Thus careful assessment of clinical features of FPLD should be considered in these patients, allowing earlier therapeutic interventions.
**Introduction**

Familial partial lipodystrophy (FPLD) is an autosomal dominant disorder characterised by abnormal distribution of subcutaneous fat accompanied by a variable degree of metabolic derangements, including marked insulin resistance and severe hypertriglyceridemia, with recurrent episodes of pancreatitis. FPLD is a heterogeneous disorder and diagnosis is predominantly based on clinical features. A number of genetic defects underlying these clinical entities have been described, but the majority of mutations are rare coding-sequence variants in either LMNA or in PPARG1-3. Although the exact numbers for estimation of prevalence are lacking, FPLD has always been considered to be rare. But whereas generalised lipodystrophies can be readily detected because of the characteristic features from birth onwards, lipodystrophy in FPLD may be overlooked because of the overlap of clinical phenotype with the more common presentation of type 2 diabetes mellitus.

The identification of FPLD patients carries distinct therapeutic consequences. Patients with FPLD require intensive measures to prevent pancreatitis and early cardiovascular disease and may benefit from leptin replacement therapy. Furthermore, identification of FPLD patients may lead to the discovery of novel causative genes that may lead to new treatment targets and improve our understanding of the physiological mechanisms underlying lipodystrophy.

In the present study we aimed to identify novel cases of FPLD in non-obese patients (BMI ≤27 kg/m²) with type 2 diabetes mellitus and marked insulin resistance, arbitrarily defined as total insulin dose ≥100 U/day.

**Methods**

In the databases of three diabetic outpatient clinics (n=5,221) we searched for patients >18 years old with type 2 diabetes mellitus, a normal to moderately elevated BMI (≤27 kg/m²) and marked insulin resistance, defined as the use of insulin ≥100 U/day. Medical records from patients selected from the databases were first screened for eligibility. Patients unable to visit the study centre because of a poor medical condition as well as patients with any other explanation for severe insulin resistance (e.g. patients with HIV-induced lipodystrophy) were excluded from the study. Patients fulfilling all inclusion and exclusion criteria were invited to our research clinic. Here, the participant's complete medical history was evaluated and fasting blood samples were drawn for biochemical analysis and DNA extraction. All participants underwent a complete physical examination with careful assessment of the presence of FPLD stigmata. Upper arm fat was
assessed by skinfold thickness measurement and interpreted using normal ranges, adjusting for age and sex. The combination of marked insulin resistance together with skinfold thickness <10th percentile was considered to be compatible with the phenotypical diagnosis of lipodystrophy. The study was approved by the institutional review board of the Academic Medical Centre in Amsterdam, the Netherlands. All participants gave written informed consent.

Genomic DNA was isolated from blood samples containing EDTA. The coding exons of PPARG, CIDEC and LMNA (exon 8–9) plus intron–exon boundaries were sequenced using a BigDye Terminator v 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3730 DNA analyser (Applied Biosystems). Primer sequences are available upon request. Functional analysis of the novel variant was performed as described previously by our group.

Figure 1  Patient selection

5221 Diabetic patients

34 Patients assessed for eligibility
17 Female
17 Male

Inclusion criteria:
>18 Years
Type 2 diabetes mellitus
BMI <27 kg/m²
>100 U insulin/day

24 Patients invited for participation
11 Female
13 Male

10 Excluded
2 HIV-positive
7 Severe co-morbidity
1 Acromegaly

Dropout
8 Were not willing to participate
4 Non responders

12 Patients included
6 Female
6 Male
Results

From a total of 5,221 patients with a diagnosis of diabetes, 24 patients met all the inclusion and exclusion criteria. Of these 24 patients, 12 were willing to participate (figure 1). Five of the 12 non-obese patients with marked insulin resistance had a lipodystrophic phenotype (table 1). Thus our selection criteria increased the likelihood of identifying FPLD >400-fold, from 5/5,221 to 5/12. The characteristics of the study participants are described in table 1. Participants S2 and S5 were included in the study despite having a BMI >27 kg/m² at the

Figure 2  MRI tissue scans of subjects with Y151C mutation

T1 weighted images were obtained from mutant and son showing moderate lipoatrophy at different sites. A: Scans of the neck showing a layer of subcutaneous (sc) fat measuring 2.2 cm and 2.3 cm. B: Cross-section at the gluteal region, showing sc fat measuring 2.1 cm and 1.4 cm. C: Cross-section at the level of upper leg region, showing a dorsal layer of sc fat measuring 0.7 cm and 0.5 cm.
centre visit, as eligibility was based on data from the patient’s medical records which, for both participants showed a BMI ≤27 kg/m². Two of the patients with a lipodystrophic phenotype (S11 and S12) had previously been diagnosed with FPLD confirmed by mutations in PPARG (R194W) and LMNA (R482W) (E. S. Stroes, unpublished observations). The third participant (S8) had a history of systemic lupus erythematosus and chronic corticosteroid use, which in itself may explain the severe insulin resistance and abnormal skinfold thickness measurement.

The fourth patient (S9) had clinical features of FPLD, including lipodystrophy, but no mutations in PPARG, LMNA or CIDEC. DNA analysis of the fifth patient (S10) resulted in the identification of a novel functional mutation in PPARG, establishing the diagnosis of FPLD.

### Table 1  Patient’s Characteristics

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<th>Patients without a lipodystrophic phenotype</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
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<td>25</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>CVD</td>
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<td>-</td>
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<td>Triglycerides (mmol/L)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4</td>
<td>9.9</td>
<td>1.3</td>
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<td>Leptin (ng/mL)</td>
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<tr>
<td>Mutation LMNA/PPARG/CIDEC</td>
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</table>

F: female, M: Male, BMI: Body mass index, PCOS: polycystic ovary syndrome, SEA: South East Asian, Af: African, NeE: North Eastern Europe, EE: Eastern Europe, Turk: Turkish, UK: United Kingdom. <sup>a</sup> Highest triglyceride levels documented, <sup>b</sup> Reference value for women 0.00-8.0 for men 20.0-90.00.
The index patient was a 61 year old female of Dutch ancestry who participated in the Olympics as an athlete. Menarch occurred at age 14, followed by regular menstrual cycles. She gave birth to a healthy son and daughter at the age of 23 and 28 respectively. At the age of 43 she was diagnosed with hypertension and hypertriglyceridemia with eruptive xanthomas. At the age of 49 she underwent a partial pancreatectomy following an episode of necrotizing pancreatitis. Shortly thereafter she developed diabetes mellitus, which was originally attributed to insulin deficiency after a pancreatectomy. At the age of 60 she underwent endovascular stent graft placement because of a significant atherosclerotic stenosis in the iliac tract. At the age of 57 the proband was referred to our lipid clinic for management of uncontrolled hypertriglyceridemia despite a combination of fibrate and statin therapy. On physical examination she was lean (BMI: 22 kg/m²).
Figure 3 The FPLD-associated Y151C mutation reduces DNA binding, and hence transcripational activity of PPARγ

a: The electropherogram tracing shows both alleles from the proband (Y151C; PPARγ2 numbering) compared with corresponding genomic DNA sequence from a healthy subject. The position of the mutation in the DBD is indicated by the arrow. Nucleotide and amino acid sequence is shown below the electropherogram tracings. b: Family pedigree. A diagonal indicates not genotyped. Half-filled symbols indicate subjects with the heterozygous Y151C mutation. Unaffected subjects are indicated by unfilled symbols. The proband is indicated by an arrow. c: Left panel: Global structure of the PPARγ-RXRα-DNA complex. The DBDs and Ligand binding domains (LBDs) of PPARγ and RXRα are shown, with the zinc ions in the PPARγ DBD. Square box indicates the region magnified in right panel. Right panel: PPARγ-DNA interaction. Magnified view of helices of PPARγ-DBD localized around the zinc ions, with Y151 indicated in stick format. Starting structure of PPARγ was adapted from the Protein Data Bank entry 3DZY. Figures were generated using PyMOL 0.99rc6 open source software. d: Impaired DNA binding of Y151C. In vitro translated RXRα or PPARγ2 (WT or mutant) proteins were incubated with [32P]-labelled probe in absence or presence of 5x unlabelled probe (WT or mutant) as indicated. Protein-DNA complexes were separated from unbound DNA on non-denaturing SDS-polyacrylamide gels and visualized by autoradiography of dried gels. e: The FPLD3-associated Y151C mutation reduces the transcriptional activity of PPARγ2. U2OS cells were transfected with expression vector encoding PPARγ2 WT or PPARγ2 Y151C respectively, and 3XPPRE-tk-Luc reporter (left panel) or a Fabp4-Luc reporter (right panel). Activation of the luciferase reporter, in the absence or presence of 1 μM rosiglitazone, is expressed as fold induction over that with empty vector in the absence of ligand, after normalisation for Renilla luciferase activity. Results are averages of at least 3 independent experiments assayed in duplicate ± standard error of the means. f: The FPLD3-associated Y151C mutation fails to display dominant negative activity. U2OS cells were transfected with 3xPPRE-tk-Luc reporter and incubated with different concentrations of rosiglitazone, as indicated. wt PPARγ2 (black bars), wt PPARγ2 plus an equal amount of wt PPARγ2 (light grey bars), wt PPARγ2 plus the Y151C mutant (dark grey bars), or wt PPARγ2 plus the dominant negative L496A/E499A mutant (white bars) and incubated with different concentrations of rosiglitazone, as indicated. Reporter activities are presented relative to the activity of wt PPARγ2 in the presence of 1 μM rosiglitazone. Results are averages of at least 3 independent experiments assayed in duplicate ± standard error of the means.

Figure 4 A novel heterozygous FPLD3-associated mutation in the PPARG gene

A novel heterozygous FPLD3-associated mutation in the PPARG gene. Alignment of the amino acid sequence surrounding Y151C in human PPARγ2 with other PPARs and nuclear receptors showing that tyrosine 151 is completely conserved amongst species. Also indicated are the zinc-coordinating cysteine residues.
with a blood pressure of 145/70 mmHg. Laboratory analysis after an overnight fast showed a total cholesterol of 3.25 mmol/L (126 mg/dL), triglycerides of 36.7 mmol/L (3251 mg/dL), HDL-c 0.7 mmol/L (27 mg/dL) and apolipoproteinB levels of 0.84 mmol/L (84 mg/dL), consistent with severe chylomicronemia. Post-heparin testing for lipoprotein lipase (LPL) activity showed normal LPL activity and no genomic DNA sequence changes were seen in the LPL gene. Glycated hemoglobin was 8.9% and insulin levels were elevated indicating insulin resistance as the primary cause for the presence of diabetes mellitus. The start of multiple daily injections of insulin totaling 140 U/day resulted in improved glycaemic control. On physical examination there was a clear excess of subcutaneous fat on the face, neck, trunk and abdomen with a lack of subcutaneous fat on the extremities. Triceps skinfold was <5th percentile even as upper arm fat area. Magnetic Resonance Imaging (MRI) tissue scans confirmed these findings showing excessive and relatively symmetrical deposition of subcutaneous fat on the face neck and upper trunk with disproportionate depletion of subcutaneous fat in the lower body (figure 2). No other abnormalities related to FPLD were observed in this patient, there was no acanthosis nigricans or hirsutism. The proband’s mother, deceased at the age of 84, had a history of hypertriglyceridemia and hypertension. The proband’s youngest sister is 50 years old, with lipodystrophy, hypertriglyceridemia and hypertension. She had no diabetes mellitus but fasting glucose (5.1 mmol/L) and insulin (138 pmol/L) levels suggested the presence of insulin resistance. Furthermore, the plasma glucose after an oral glucose load of 75 grams was 8.3 mmol/l, indicating the presence of impaired fasting glucose. The proband’s son is 39 years old with prominent musculature and accumulation of subcutaneous facial, neck and abdominal fat despite normal anthropometry. MRI tissue scans showed lipodystrophy at various anatomical sites (figure 2). He also had a history of hypertriglyceridemia and hypertension. He had no diagnosis of type 2 diabetes mellitus but fasting plasma glucose (4.9 mmol/L) and insulin levels (90 pmol/L) suggested the presence of insulin resistance. We have no clinical data of the proband’s nephew.

S10 had a heterozygous A-to-G mutation in exon 5 of the PPARG gene (figure 3a), resulting in a Y151C substitution in the PPARγ2 isoform. DNA sequence analysis of family members of the index patient showed the same mutation in the patient’s son, sister and nephew (figure 3b). Tyrosine 151 is located in the DNA-binding domain (DBD) of PPARγ and is completely conserved amongst species and well conserved among nuclear receptors (figure 4). Crystal structure studies of the PPARγ–retinoidX receptor, α (RXRa) heterodimer bound to DNA indicate that Y151 is in direct contact with the DNA backbone (figure 3c). In vitro experiments showed the Y151C mutant to
Discussion

In the present study, we show an increase in the incidence of FPLD in non-obese patients with type 2 diabetes mellitus and marked insulin resistance. Characterisation of 12 participants fulfilling these criteria resulted in the identification of five patients with lipodystrophic features. In three patients the diagnosis of FPLD was supported by mutations in \textit{PPARG} or \textit{LMNA}, with one patient harbouring a novel heterozygous \textit{PPARG} mutation (Y151C). These data indicate that thorough evaluation for FPLD should be considered in non-obese patients with marked insulin resistance. However, the numbers in our study may deviate from the actual incidence of FPLD. First, the small number of eligible patients willing to participate in the study may have resulted in a positive 'selection' bias. Second, our stringent inclusion criteria may have increased the false-negative rate. The aim of the present study was to find an effective method to select rare cases of FPLD from a large group of individuals with the common type 2 diabetes mellitus. To effectively increase the chances of identifying FPLD, we aimed to select patients with an unusual phenotype, namely non-obese patients with marked insulin resistance. Our inclusion criteria may, however, have been too stringent as neither absence of marked insulin resistance nor the presence of obesity excludes the presence of FPLD. It should be noted that lipodystrophy may be more easily recognised in patients with increased BMI because of the accentuated differences in subcutaneous fat in extremities versus trunk when patients become obese. Thus, although our selection criteria may serve as a guideline, the presence of other typical features of FPLD, in any patient, should still alert clinicians to the possibility of lipodystrophy. To date, 15 different FPLD-associated mutations in the coding region of \textit{PPARG} have been reported\textsuperscript{9}. Here we identified a novel heterozygous tyrosine-to-cysteine mutation located in the region of the \textit{PPARG} gene encoding the DBD (Y151C). The mutation was shown to result in reduced DNA binding and reduced transcriptional activity, while direct dominant negative activity was absent. Tyrosine 151 is located in the DBD of PPAR\textgamma and is completely conserved amongst species and within the nuclear receptor superfamily. Interestingly, mutation of the analogous position in the androgen receptor (Y571) to histidine or cysteine has been associated with various forms of the androgen insensitivity syndrome (Androgen Receptor Gene Mutations Database; http://androgendb.mcgill.ca). Like the PPAR\textgamma Y151C mutant reported in this study, the androgen receptor Y571H mutant displayed impaired DNA-binding capacity and hence reduced transcriptional activity\textsuperscript{10}. These
findings underscore the importance of this conserved tyrosine residue in DNA
binding by nuclear receptors.

In summary, our results indicate that careful assessment of the clinical features of
FPLD should be considered in non-obese patients with marked insulin resistance,
allowing earlier therapeutic interventions.
Reference List


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