

Prevalence of Mutations in *AGPAT2* Among Human Lipodystrophies

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Berardinelli-Seip congenital lipodystrophy (BSCL) is a heterogeneous genetic disease characterized by near absence of adipose tissue and severe insulin resistance. We have previously identified mutations in the seipin gene in a subset of our patients' cohort. Recently, disease-causing mutations in *AGPAT2* have been reported in BSCL patients. In this study, we have performed mutation screening in *AGPAT2* and the related *AGPAT1* in patients with BSCL or other forms of lipodystrophy who have no detectable mutation in the seipin gene. We found 38 BSCL patients from 30 families with mutations in *AGPAT2*. Three of the known mutations were frequently found in our families. Of the eight new alterations, six are null mutations and two are missense mutations (Glu172Lys and Ala238Gly). All the patients harboring *AGPAT2* mutations presented with typical features of BSCL. We did not find mutations in patients with other forms of lipodystrophies, including the syndromes of Lawrence, Dunnigan, and Barraquer-Simons, or with type A insulin resistance. In conclusion, mutations in the seipin gene and *AGPAT2* are confined to the BSCL phenotype. Because we found mutations in 92 of the 94 BSCL patients studied, the seipin gene and *AGPAT2* are the two major genes involved in the etiology of BSCL. *Diabetes* 52:1573–1578, 2003

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BSCL, Berardinelli-Seip congenital lipodystrophy; FPLD, familial partial lipodystrophy; LPA, lysophosphatidic acid.

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Generalized lipodystrophy belongs to the heterogeneous group of syndromes characterized by alterations in body fat distribution and insulin resistance. These also display clinical features of acanthosis nigricans, hyperandrogenism, muscular hypertrophy, hepatomegaly, and at the biological level, altered glucose tolerance or diabetes and hypertriglyceridemia (1). Syndromes of lipodystrophy are classified according to the pattern of lipodystrophy being either localized or generalized (2–4). In the generalized form, both subcutaneous and visceral adipose tissues are near absent. Two phenotypes are distinguished according to the age at onset of lipoatrophy: the congenital form or Berardinelli-Seip congenital lipodystrophy (BSCL) with absence of adipose tissue present from birth or occurring in early infancy (OMIM #26970) and the delayed form or Lawrence syndrome where lipoatrophy occurs at a later age (1–3,5).

BSCL is transmitted as an autosomal recessive trait, while the genetic basis of the Lawrence syndrome is still a matter of debate (2–4). The positional cloning strategy revealed that BSCL is a heterogeneous genetic disease with, until now, two genes identified (6,7). Other gene(s) might be inferred from genetic studies of a few families where the disease is not linked to the two identified loci (8,9). The first gene, *BSCL2*, that we identified on chromosome 11q13, encodes the protein “seipin” of unknown function and without similarities with known proteins (6). Its alteration causes the disease in families originating from Northern Europe, Portugal, and the Middle East (6,8–10). Recently, Agarwal et al. (7) identified a second gene on chromosome 9q34, *AGPAT2*, that when mutated is responsible for the disease in most of their family cohort. *AGPAT2* encodes the enzyme 1-acyl-glycerol-3-phosphate acyltransferase- β , which is involved in the synthesis of triglycerides (11,12). This enzyme catalyzes the conversion of 1-acylglycerol-3-phosphate (lysophosphatidic acid [LPA]) to 1,2-diacylglycerol-3-phosphate (phosphatidic acid) (11–13).

In the present study, we searched for mutations in *AGPAT2* and its isoform *AGPAT1* in patients presenting with BSCL and other forms of lipodystrophy without a detectable mutation in the seipin gene. We found muta-

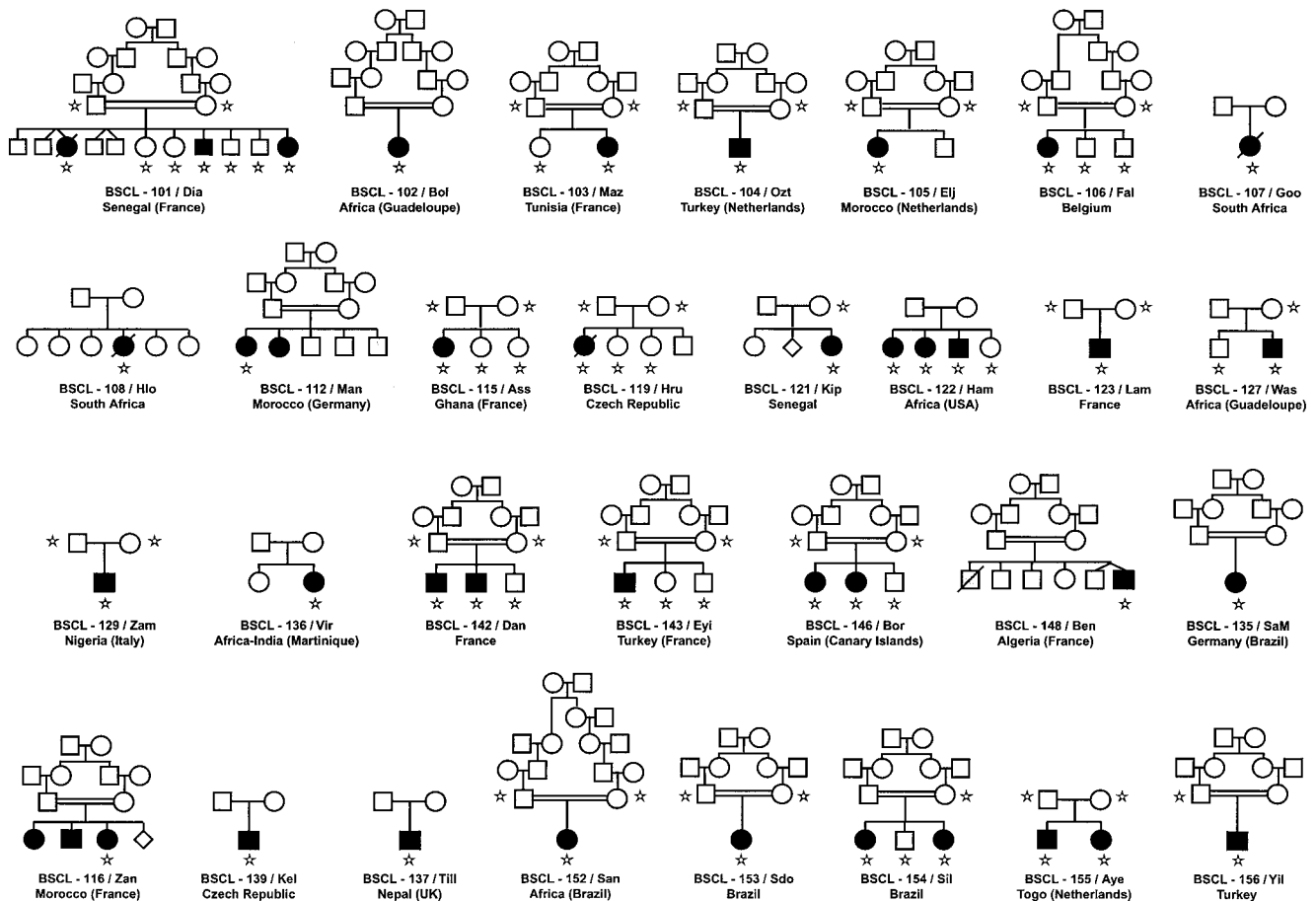


FIG. 1. Pedigrees of families with BSCL disclosing mutations in *AGPAT2*. Open symbols, unaffected individuals; filled symbols, affected individuals; gray symbols, individuals with uncertain phenotype; diagonal slash, deceased patients. Stars denote subjects for which DNA was available. When the country of origin is known to be different from country where the patient is living, this latter is indicated between brackets.

tions in *AGPAT2* in all patients presenting with BSCL (38 patients from 30 families) but two. Eleven different molecular alterations were seen, essentially in exons 2–6. Pedigrees of these families are shown in Fig. 1, and the *AGPAT2* mutations detected are reported in Table 1.

Three of the mutations that are present in several families have been previously described (7). The nonsense mutation Arg68X that was detected in two families (7) is present here in six families originating from Belgium, France, Morocco, and Turkey. Haplotype analysis using biallelic markers located within the gene revealed that all the patients from this study are homozygous for the same disease-associated allele, suggesting that these families might be related due to a founder effect (allele I, Fig. 2).

The deletion of the exons 3 and 4 described in a European family of Portuguese ancestry (7) has been found in three families living in the Canary Islands and Brazil that might be related from haplotype analysis (allele II, Fig. 2). Sequencing of the genomic DNA fragment from these patients allowed us to determine the nature of the alteration. This is a 1,036-bp deletion that encompasses the 50th nucleotide of exon 3 to nucleotide +534 within intron 4. This specific deletion was not present in the heterozygous state in the remaining families.

The intron 4 acceptor splice site mutation, IVS4 –2A→G, was previously observed in five families (7). In our study, this mutation is present in 13 families, all of

African ethnicity including two families originating from Sub-Saharan Africa. Haplotype analysis revealed three disease-associated alleles, suggesting the recurrence of independent mutational events (alleles IV, V, and VI, Fig. 2). In the report from Agarwal et al. (7), the five families disclosing this splice mutation were suspected from haplotype analysis to be related. They all are of African origin living in the Caribbean or U.S., and it is likely that most of them are descended from slaves deported from Africa and therefore related to one set of our families.

The eight other molecular alterations have not yet been reported. Three novel molecular alterations found in *AGPAT2* are splice-site mutations. Two of these mutations affect nucleotides at position –1 or –2 in the consensus sequence of the intron 1 or 2 acceptor splice sites. These nucleotides belong to a strictly conserved nucleotide consensus sequence for RNA (14). Mutations at this location usually induce the skipping of the following exon. We also found a unique variant affecting a nucleotide located in the consensus sequence of the intron 5 donor splice site (position +2: T→G). Mutations at this location usually induce the skipping of the previous exon and should here result in the skipping of exon 5. Therefore, this mutation should generate the same protein product as the splice mutation IVS4–2A→G that we have shown, after RT-PCR and sequencing, to induce exon 5 skipping. This should lead to a shift of the reading frame after amino acid 196

TABLE 1
Mutations found in AGPAT2

Gene position	Nucleotide* alteration	Predicted amino acid change	no.	Initials	Birth/ death	Sex	Patients/families			Origin (living country)
							Age at onset	Status	Inbreeding	
Intron 1 Exon 2	IVS1 -2A → G	F60fsX102	136	Vir V	1975	F	<21 m	het	-	Africa-India (Martinique)
	G ₁₉₄ → A C ₂₀₂ → T	Trp65X Arg68X	116 105 106	Zan A Eli L Fal V	1976 1991 1974	F F F	Birth Birth Birth	hom hom hom	+ + +	Morocco (France) Morocco (Netherlands) Belgium
Intron 3 Exons 3-4	IVS3 -1G → C del 317-588	delleu165-Gin196 Gly106fsX188	112 123 143 148 135 146.1	Man H Lam J Eyl F Ben M Sam G Bor H	1978 1997 1996 2001 1994 1986	F M M M F F	Birth Birth 3 m 3 m Birth Early childhood	hom hom hom hom hom hom	+ - + + + +	Morocco (Germany) France Turkey (France) Algeria (France) Germany (Brazil) Spain (Canary Islands) Spain (Canary Islands)
			146.2 153 154.1 154.2	Bor V Sdo G Sil GN Sil BN	1988 2000 1994 1999	F F F F	4 m 4 m 1 m Birth	hom hom hom hom	+ + + +	Brazil Brazil Brazil Turkey (Netherlands)
Exon 4	G ₅₁₄ → A	Glu172Lys	104	Ozt U	1990	M	Birth	hom	+	Czech Republic
Intron 4	IVS4 -2 A → G	F109fsX452	139	Kel J	1997	M	Birth	hom	-	
			101.1	Dia H	1972-99	F	Birth	hom	+	Senegal (France)
			101.2	Dia I	1984	M	Birth	hom	+	Senegal (France)
			101.3	Dia N	1987	F	Birth	hom	+	Senegal (France)
			103	Maz N	1990	F	Birth	hom	+	Tunisia (France)
			115	Ass L	1983	F	Birth	hom	-	Ghana (France)
			121	Kip PM	1996	F	Birth	hom	-	Senegal
			122.1	Han L	1962	F	Birth	hom	-	Africa (U.S.)
			122.2	Han L	1965	F	Birth	hom	-	Africa (U.S.)
			122.3	Han HD	1967	M	Birth	hom	-	Africa (Guadeloupe)
			127	Was X	1977	M	Birth	hom	-	Africa (Guadeloupe)
			129	Zam J	1993	M	Birth	het	-	Nigeria (Italy)
			136	Vir V	1975	F	<21 m	het	-	Africa-India (Martinique)
			102	Bol J	1975	F	Birth	hom	+	Africa (Guadeloupe)
			107	Goou N	1974-92	F	Birth	hom	+	South Africa
			108	Hlo I	1943-92	F	Early childhood	hom	-	South Africa
			152	San T	1989	F	3 m	hom	+	Africa (Brazil)
			155.1	Aye I	1993	F	Early childhood	hom	-	Togo (Netherlands)
			155.2	Aye S	2000	M	Birth	hom	-	Togo (Netherlands)
Exon 5	A ₆₄₅ → T IVS5 +2 T → G	Lys216X F109fsX452	156 142.1	Yil M Dan L	1999 1989	M M	Birth Birth	hom hom	+	Turkey
Intron 5			142.2	Dan M	1997	M	Birth	hom	+	France
			137	Thl A	2000	M	<6 w	hom	+	France
Exon 6	C ₆₇₆ → T C ₇₁₂ → G	Gln226X Ala238Gly	137	Thl A	2000	M	<6 w	hom	-	Nepal (U.K.)
			119	Hru P	1980	F	<2 y	het	-	Czech Republic

*Nucleotide designation commencing 1 at position 67 (translation start) of Genbank entry NM_00642 (AGPAT2). het, heterozygote; hom, homozygote for the mutation; m, months; w, weeks; y, years.

			I	II	III	IV	V	VI
Prom:	G-67	>	C					
E1[38-40]:	wt	>	ins[TGC]					
IVS1 +6:	G	>	A					
IVS1 -60:	G	>	C					
E2:	C202	→	T					
E3-E4:	wt	→	Δ[E3-E4]					
E4:	G514	→	A					
IVS4 -2:	A	→	G					
E6:	C847	>	T					
			G	G	G	G	G	G
			wt	wt	ins[TGC]	wt	wt	wt
			G	G	G	G	G	G
			C	C	G	C	C	C
			T	C	C	C	C	C
			wt	Δ[E3-E4]	wt	wt	wt	wt
			G	G	A	G	G	G
			A	A	A	G	G	G
			C	C	C	C	C	T
			105	146	104	101	102	115
			106	153	139	127	103	121m
			112	154		129m	107	152
			123			136m	108	155m
			143				115m	
			148				121p	
							122	
							155p	

FIG. 2. Disease-associated alleles inferred from haplotype analysis in each group of patients disclosing the same mutation. *Left*: Bi-allelic markers and, in bold characters, DNA alterations; filled boxes represent the allele with at the top, roman letters indicate the allele number and at the bottom, the number of each carrier patient. *Right*: For heterozygous patients, m and p indicate the maternal and paternal allele, respectively. Three mutations are imbedded in the same carrier alleles, suggesting that each of these mutations represents a unique mutational event and that the patients might be related (respectively, in exon 2: nonsense mutation [C₂₀₂→T] in allele I, deletion of exons 3 and 4 in allele II, and in exon 4: missense mutation [G₅₁₄→A] in allele III). Conversely, three different alleles, IV, V, and VI, are distinguished among patients disclosing the splice mutation IVS4-2A→G, suggesting the recurrence of independent mutational events.

and cause the addition of 102 novel amino acids to the COOH-terminal end of the mutant protein (Gln196fsX452). The unique COOH-terminal end of the mutant protein shows no homology to previously identified protein sequences.

Three novel nonsense mutations were identified. A nucleotide change (G₁₉₄→A) observed in exon 2 leads to the substitution of the tryptophane residue at position 65 by a stop codon (Trp65X). In exon 5, the A₆₄₅→T change replaces the lysine residue at position 216 by a stop codon (Lys216X). In exon 6, a A₆₄₅→T change replaces the glutamine residue at position 226 by a stop codon (Gln226X).

Only two missense mutations have been detected. The Glu172Lys substitution has been found in patients from the Czech Republic and Turkey who might be related from haplotype analysis (allele III, Fig. 2). It affects the glutamic acid residue within the motif EGTR that is conserved through different species and in the five AGPAT isoforms (13,15). This residue is also present in other acyltransferases that do not use LPA as substrate, such as glycerol-3-phosphate acyltransferase (G3PAT), dihydroxyacetonephosphate acyltransferase, and tafazzin (13,16) (Fig. 3). Using the *E. coli* G3PAT as a model enzyme, site-specific mutagenesis experiments have previously shown that the invariant glutamic acid residue (and the next glycine) within this motif is essential for the catalytic function involved in binding the substrate (16). The Glu172Lys mutation therefore might alter the binding of LPA. The other missense mutation, Ala238Gly, is unique in a family from the Czech Republic. It affects the alanine residue at position 238 that is not conserved through species and human isoforms (13,15). However, the identification of a mutation at the next alanine residue, Ala239Val, in

another patient with BSCL (7) suggests that these two adjacent alanine residues might be part of an important site for regulatory or functional specificity of the enzyme.

Therefore, most of the mutations detected in BSCL patients are null mutations that induce synthesis of aberrant or shorter nonfunctional proteins or that target the mRNA for degradation by a mechanism involving a quality-control step. They likely affect triacylglycerol synthesis in adipose tissue and may cause lipodystrophy by depleting the adipocytes of triglycerides (11-13). With the exception of patients no. 119 and 129 for whom the second mutation has not yet been identified, patients with BSCL are either homozygous for a specific mutation or compound heterozygous in accordance with an autosomal recessive pattern of inheritance. These data provide further evidence that mutational events in *AGPAT2* are responsible for BSCL in this subset of patients.

All the other nucleotide variations that we detected in *AGPAT2* are polymorphisms that are silent or that have been found in 96 control subjects. Some of these polymorphisms are exonic: in exon 1, insertion (TGC)₃₈₋₄₀, insertion of Leu₁₄; in exon 2, (C₁₈₉→T), Ile63, (C₂₂₉→T), and Arg77Cys; in exon 4, (C₅₆₁→T), and Gly187; and in exon 6, (C₇₀₂→T) and Ser234. The other are located in the promoter region, G-29→C and deletion (AGCGGG)₅₅₋₅₀; in intron 1, IVS1 + 6 G→A and IVS1-60 G→C; and in the 3'untranslated region, 837/+10 C→T and 837/+48 T→C.

Among the 94 patients with BSCL that we have studied so far, all but 2 patients have disclosed mutations in either the seipin gene (54 patients, 30 families) (6 and personal communication) or in *AGPAT2* (38 patients, 30 families). The haplotype analysis in the two families without muta-

			↓	
AGPAT2	160	MVREN LK V W I Y P	E G	TRNDNGDLLPFKKG ₁₈₇
AGPAT1	166	LLTQDVRVWVFP	E G	TRNHNGSMLPFKRG ₁₉₃
AGPAT3	164	DYPEYMWFLLYC	E G	TRFTE TKHRVSM EV ₁₉₁
AGPAT4	164	DYPEKYFFLIHCE	E G	TRFTEKKHEISMV ₁₉₁
AGPAT5	161	DAGTPMYLVIFP	E G	TRYNPEQTKVLSAS ₁₈₈
G3PAT	303	LLRQQQFLEIFL	E G	TRSRSGKTS CARAG ₃₃₀
DHAPAT	231	LRNGYAPVEFFL	E G	TRSRSAKTLTPKFG ₂₅₈
TAZ	168	KLNHGDWVHIFP	E G	KVNMSS EFLRFWGI ₁₉₆

FIG. 3. Amino acid sequence alignment of human enzyme isoforms encoded by AGPAT2, -1, -3, -4, and -5; glycerol-3-phosphate acyltransferase (G3PAT); dihydroxy-acetonephosphate acyltransferase (DHAPAT); and tafazzin (TAZ). The bold characters indicate positions that have a single, fully conserved residue. The arrow points out the position of the missense mutation at glutamic acid residue 172 (Glu172Lys), which is highly conserved through AGPAT isoforms and acyltransferases.

tion in these two genes were not informative enough, so we cannot exclude the possibility of large rearrangements or point mutations affecting the promoter or other potential regulatory sequences. Alternatively, these data might suggest that alterations in a third gene are responsible for the disease in a few cases as previously suggested (8,9). In the present study, we also searched for molecular alterations in *AGPAT1* that encodes the enzyme isoform- α (12). At the physiological level, both enzyme isoforms- α and - β seem to exert similar functions (13,17). However, they might have a different pattern of expression since *AGPAT1* mRNA is rather ubiquitous while *AGPAT2* mRNA might be predominant in adipose tissue (7,11–13). The systematic sequencing of the seven exons of *AGPAT1* revealed no mutation in patients affected with BSCL. The other candidates tested for the lipodystrophy locus were the *LPIN* genes, since mutations in *Lpin1* induce a lipotrophic phenotype in the fld mouse model. However, the genetic analysis performed in two consanguineous families excluded the implication of the three isoforms known in human *LPIN1*, *LPIN2*, and *LPIN3* (8). Therefore, further studies are required in these families in order to find the gene(s) responsible for the disease.

Mutations in *AGPAT2* were not detected in the 19 patients affected with Lawrence syndrome, the delayed form of generalized lipodystrophy (1,5). Since these patients originate from countries where all BSCL patients harbor mutations in either the seipin gene or *AGPAT2*, it is likely that Lawrence syndrome has a different etiology.

It is possible that nongenetic factors contribute to generalized lipodystrophy in these patients. Indeed, the occurrence of lipotrophy in Lawrence syndrome is often preceded by intercurrent illnesses or infections and may be associated with autoimmune diseases (1–3). Interestingly, autoantibodies against adipocyte membranes have been found in the serum from a patient with Lawrence syndrome (18). Similarly, in our cohort, 3 of the 10 patients tested had antibodies against adipocytes. Thus, although the etiology of Lawrence syndrome is unknown, immune mechanisms are likely to be involved.

If we consider Lawrence syndrome as a genetic disorder, the gene(s) responsible for the generalized lipodystrophy must differ from the seipin gene and *AGPAT2*. So far, molecular scanning of candidate genes including the insulin receptor (19), the IRS-1 (20), the peroxisome proliferator-activated receptor- γ (21), the type-A lamin (22), and in the present study, *AGPAT1*, did not reveal any molecular alteration. Furthermore, assuming an autosomal recessive mode of inheritance, genetic studies in 2 consanguineous families allowed exclusion of these genes as well as of 12 other candidate genes involved in insulin action and lipid metabolism (22–24). Thus, other molecular studies are required in order to determine whether any gene(s) is(are) altered in Lawrence syndrome.

We did not find mutations in *AGPAT2* and in *AGPAT1* in forms of partial lipodystrophy (3) with features resembling Dunnigan-type familial partial lipodystrophy (FPLD) (18 patients), Barraquer-Simons (6 patients), and in type A syndrome of insulin resistance (25) (32 patients). None of these patients were found to have mutations in *LMNA*, which encodes the type A lamins usually mutated in FPLD patients (2–4,22), or in the insulin receptor gene that is

mutated in a minority of patients with type A syndrome. These results indicate that a different gene (or genes) or nongenetic factors could be implicated in the development of lipodystrophy or insulin resistance in these patients.

The results of the present study confirm and extend findings from a previous cohort of patients (7) indicating that mutations in *AGPAT2* cosegregate only with the phenotype of BSCL. The identification of these mutations enhances the possibility for prenatal diagnosis and prevention of BSCL. The characterization of the mechanism by which these mutations cause the BSCL phenotype is likely to provide significant insights into the molecular basis of lipotrophy. In conclusion, BSCL is a heterogeneous genetic disease caused by mutations in the seipin gene and *AGPAT2*. Because 92 of the 94 BSCL patients that we studied disclose mutations, the seipin gene and *AGPAT2* are the two major genes involved in the etiology of BSCL.

RESEARCH DESIGN AND METHODS

Patients. We studied 115 patients in whom no mutation in the seipin gene was detected. All patients and their families gave their informed consent for genetic studies, which were approved by local institutional review committees.

A total of 40 patients from 32 families presented with BSCL with a generalized form of lipotrophy and muscular hypertrophy from birth or beginning in early infancy. Most of them presented with acromegaloïd features, acanthosis nigricans, hepatomegaly, hirsutism, insulin resistance, and hypertriglyceridemia (1–4). The biological and clinical characteristics of some of the patients have been previously reported (9,19,20,22–24).

Altogether, 19 patients (14 women and 5 men) were affected with Lawrence syndrome, i.e., generalized lipotrophy occurring from childhood or adulthood. Although delayed, the phenotype is similar to BSCL once the loss of fat has become generalized with the same clinical and metabolic features (1,3,5,9,19,22–24).

Eighteen patients presented with partial lipodystrophies with features resembling FPLD characterized by a lack of adipose tissue in the limbs, buttocks, and trunk with fat accumulation in the neck and face (2–4). These patients did not have detectable mutations in *LMNA*.

Six patients were affected with the syndrome of Barraquer-Simon characterized by cephalothoracic lipotrophy and accumulation of fat in the lower part of the body (3). Five of the six patients tested did not have detectable mutations in *LMNA*.

Thirty-two patients were diagnosed with the type A syndrome of insulin resistance (25). We verified the absence of mutations in the genes coding the type A lamins or the insulin receptor.

Mutation screening. Specific primers were designed to specifically amplify *AGPAT1* and *AGPAT2* exons and splice junctions using the website of Massachusetts Institute of Technology (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Sequence of these intronic primers is available upon request. PCR products were purified on Sephadex column and sequenced using Big Dye Terminator chemistry (Applied Biosystems). Sequence comparisons were performed using Phred Phrap Consed software. The specific *AGPAT2* 1,036-bp deletion encompassing nucleotide 50 of exon 3 to nucleotide +534 of intron 4 was searched using the triplet of primers (F₁: 5'-CGGCCTCAGTACCTCCCTCA-3'; F₂: 5'-CGAGCTGAGGACCCTTGACAC-3'; R: 5'-CGCAACTCTCCAGCACAG-3'). We analyzed splice site mutations using total RNA extracted from cultured cells obtained from the patients as a template and the SMART PCR cDNA synthesis kit (Clontech). For the first PCR, we used primers 1F₆₇: 5'-ATGGAGCTGTGGCCGTGTCT-3' and 6R₇₅₈: 5'-ATGGCTTCCAGCACCTGCAC-3' and conditions as follows: 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 65°C for 45 s, 72°C for 2 min, and a final extension at 72°C for 10 min. We then performed a nested PCR with internal primers.

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