UNIVERSITY OF ALGARVE

FACULTY OF ENGINEERING OF NATURAL RESOURCES

IDENTIFICATION OF POLYMORPHISMS AND CHARACTERIZATION OF NEW OVINE GROWTH HORMONE VARIANTS ASSOCIATED WITH MILK TRAITS IN "SERRA DA ESTRELA" OVINE BREED.

(Thesis dissertation presented to obtain the PhD degree in Biology, speciality of Population Biology)

Maria do Rosário Fernandes Marques

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FARO

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À minha ***Mãe***

THESIS PUBLICATIONS

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TÍTULO DA TESE:
Identificação de polimorfismos e caracterização de novas variantes proteicas da hormona de crescimento associadas com a produção de leite na raça ovina Serra da Estrela.

RESUMO

O presente trabalho teve como objectivos identificar e caracterizar polimorfismos genéticos no gene da hormona de crescimento (*oGH*) em ovinos Serra da Estrela e estabelecer possíveis associações entre eles e a produção e composição do leite, de modo a avaliar a aplicabilidade do gene da *GH* em selecção assistida por marcadores genéticos.
O gene da *GH* apresentou elevado polimorfismo, tendo sido preditas oito e dez variantes proteicas codificadas pelas cópias *GH2-N* e *GH2-Z*, respectivamente.
Verificou-se a ocorrência de associações significativas entre os polimorfismos encontrados e a produção e composição do leite (teores e produção de gordura e proteína). O genótipo N2+Z7 (*GH2-N*+*GH2-Z*) produziu mais 39,6 ± 7,5 litros de leite/150 dias, com maior teor em gordura e igual teor em proteína que o N5+Z2 (mais 25% da produção média das ovelhas). Resultados semelhantes foram observados nas ovelhas com o fenótipo proteico AA_N+BB_Z.
Os resultados indicam que os polimorfismos do gene da GH poderão vir a ser utilizados na selecção assistida por marcadores genéticos. Estes poderão permitir o melhoramento da produção de leite sem afectar a sua qualidade. Contudo, a resposta à selecção dependerá das condições de exploração intrínsecas a cada rebanho e nomeadamente do maneio alimentar dos animais.

Palavras-chave: Ovinos; gene da hormona de crescimento; polimorfismos; PCR-SSCP; marcadores genéticos; produção de leite.

TITLE:

Identification of polymorphisms and characterization of new ovine growth hormone variants associated with milk traits in "Serra da Estrela" ovine breed.

ABSTRACT

The objectives of the present work were the identification and characterisation of nucleotidic polymorphisms naturally occurring at the growth hormone gene (*oGH*) in "Serra da Estrela" sheep, and the establishment of associations with milk traits in order to evaluate GH as a useful candidate gene for marker assisted selection.

The *oGH* gene was found to be highly polymorphic. Polymorphisms found in coding regions allowed the prediction of eight protein variants coded by the *GH2-N* copy and ten by the *GH2-Z* copy.

Milk yield and milk composition (fat and protein contents and yields) were associated with GH polymorphisms by restricted maximum likelihood (REML) through a univariate best linear unbiased prediction (BLUP) animal model with repeated measures.

The N2+Z7 (*GH2-N*+*GH2-Z*) genotype produced 39.6 ± 7.5 litres of milk/150 days more than N5+Z2 (more 25 % than the mean milk yield of the studied population), with higher milk fat content and similar protein content. Moreover, a similar result was obtained for the protein phenotype AA_N+BB_Z ewes.

The results indicate that using GH polymorphisms as genetic markers could improve milk yield potential in "Serra da Estrela" ewes without detrimental impact on milk quality. The extent of the response, however, might depend on the environmental conditions within the flock, namely on an appropriate feeding management of the animals.

Key words: *Ovis aries*; growth hormone gene; polymorphism; PCR-SSCP; genetic markers; milk yield.

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LIST OF ABBREVIATIONS

∞	infinite
χ^2	chi-square
µl	micro litre(s)
µM	micro molar
A	adenine residue
aa	amino acid(s)
AFLP	amplified fragment length polymorphism
ALS	acid-labile subunit
ANCOSE	National Association of the Breeders of the "Serra da Estrela" Sheep
Ap-1	activator protein 1
bGH	bovine growth hormone
BLUP	Best Linear Unbiased Predictor
bp	base pair(s)
BSA	bovine serum albumin
bST	bovine somatotropin
C	cytosine residue
Ca^{2+}	calcium ion
CAAT box	consensus sequence GGCCAATCT
cAMP	cyclic adenosine 3',5'-monophosphate
c-fos	cellular *fos* oncogene
cm	centimetre(s)
CREB	cAMP-responsive element binding protein
CSPD	chemiluminescent alkaline phosphatase substrate
d	day(s)
D_1	D_1-like dopamine receptor
D_2	D_2- like dopamine receptor
DAG	1,2-diacylglycerol
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	2'-deoxynucleoside triphosphate
DRSEG	Demarcated Region of "Serra da Estrela" Cheese
EC	European Community
EDTA	potassium-ethylenediaminetetracetic acid
EGF	epidermal growth factor
ERK	extracellular-signal-regulated kinase
FAO	Food and Agriculture Organization of the United Nations
FBAT	Family-Based Association Tests
FL(s)	flock(s)
Fwd	forward
g	gram (s)
G	guanine residue
GABA	γ-aminobutyric acid
GalR	galanin receptor
GC	glucocorticoid(s)
gGH	goat growth hormone

GH	growth hormone
GH1	growth hormone codified by the ovine *GH1* gene copy
GH1	growth hormone gene copy of the ovine Gh1 allele
Gh1	non-duplicated ovine growth hormone gene allele
Gh2	duplicated ovine growth hormone gene allele
GH2-N	5' growth hormone gene copy of the ovine *Gh2* allele
GH2-N	growth hormone codified by the ovine *GH2-N* gene copy
GH2-Z	3' growth hormone gene copy of the ovine *Gh2* allele
GH2-Z	growth hormone codified by the ovine *GH2-Z* gene copy
GHBP	growth hormone binding protein
GHMM	generalized hidden Markov models
GHR	growth hormone receptor
GHRH	growth hormone-releasing hormone
GHRHR	growth hormone-releasing hormone receptor
GHRP	growth hormone-releasing peptide
G_I	inhibitory guanyl nucleotide-binding protein
GIP	glucose-dependent insulin-inducing peptide
GLUT1	erythrocyte-type glucose transporter
GLUT2	liver-type glucose transporter
GLUT4	insulin-responsive glucose transporter
GLUT5	intestinal-type glucose transporter
GR	glucocorticoid receptor
Grb10	growth-factor-bound protein 10
Grb2	growth-factor-bound protein 2
G_S	stimulatory guanyl nucleotide-binding protein
ha	hectare
hGH	human growth hormone
hGHBP	human growth hormone binding protein
h*GH-N*	human growth hormone gene copy N
hGHRH	human growth hormone-releasing hormone
HMM	hidden Markov models
HWE	Hardy-Weinberg equilibrium
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding proteins
INE	National Institute of Statistics of Portugal
JAK	Janus tyrosine kinase
kb	kilo base pair(s)
KCl	potassium chloride
kg	kilogram(s)
l	litre(s)
LCR	locus control region
LPA	lysophosphatidic acid
M	molar
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAS	marker assisted selection
MEK	MAP/ERK kinase
mg	milligram
$MgCl_2$	magnesium chloride
MGF	mammary gland factor; STAT5

min	minute(s)
MKP1	MAPK phosphatase 1
mm	millimetre(s)
MME	mixed model equations
mRNA	messenger ribonucleic acid
***Msp*I**	restriction endonuclease *Msp*I
MTDFREML	multiple-trait derivative free restricted maximum likelihood
MY	milk yield
My	Million years
N	North
NaOH	sodium hydroxide
nd	no date; not determined
NEFA	non-esterified fatty acids
NPY	neuropeptide Y
NPY-Y	neuropeptide Y receptor
NRE	negative regulatory element
nt	nucleotide(s)
OB	leptin
ob	leptin gene
ºC	degrees Celsius
oGH	ovine growth hormone
PACAP	pituitary adenylate cyclase-activating polypeptide
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDO	protected denomination of origin
PDO	Protected Denomination of Origin
PEPCK	phosphoenolpyruvate carboxykinase
pGH	pig growth hormone
PKA	protein kinase A
PKC	protein kinase C
PL	placental lactogen
polyA	polyadenylation
POU	Pit-1/Oct-1/Unc-86
POU1F1	pituitary-specific transcription factor 1
PRL	prolactin
PRLR	prolactin receptor
PROP1	prophet of Pou1F1
QTL	quantitative trait loci
raf	ras oncogene
RAPD	random amplified polymorphic DNA
RAR	retinoic acid receptor
ras	ras oncogene
REML	Restricted Maximum Likelihood
Rev	reverse
RFLP	restriction fragment length polymorphism
rGH	rat growth hormone
RNA	ribonucleic acid
s	second(s)
SE	Standard error
SH2	Src homology 2

Shc	SH2-containing protein
SHP	SH2 domain-containing protein tyrosine phosphatases
SIRPα1	signal regulatory protein-α1
SNP	single nucleotide polymorphism
SOCS	suppressors of cytokine signalling
Sos	son-of-sevenless
Sp1	stimulating protein 1
Spi2.1	serine protease inhibitor 2.1 gene
SRIF	somatotropin release-inhibiting factor; somatostatin
SSCP	single-strand conformation polymorphism
sst	SRIF receptor
ST	somatotropin; used as synonymous of exogenously administrated GH
STAT	signal transducers and activators of transcription
T	thymine residue
T3	3,5,3'-triiodothyronine; thyroid hormone
T3R	thyroid hormone receptor
TATA box	consensus sequence TATAAAT
TBE	Tris/borate/EDTA
TMY	total milk yield
TRE	thyroid hormone response element
UTR	untranslated region
V	Volt(s)
vs.	*versus*
W	Watt(s); West
WAP	whey acidic protein
YY1	yin and yang factor 1
Zn-15	zinc finger protein
Zn15/Zn16	zinc finger transcription factor

LIST OF AMINO ACIDS

One- and three- letter symbols for amino acids:

A	**Ala**	Alanine
C	**Cys**	Cysteine
D	**Asp**	Aspartic acid
E	**Glu**	Glutamic acid
F	**Phe**	Phenylalanine
G	**Gly**	Glycine
H	**His**	Histidine
I	**Ile**	Isoleucine
K	**Lys**	Lysine
L	**Leu**	Leucine
M	**Met**	Methionine
N	**Asn**	Asparagine
P	**Pro**	Proline
Q	**Gln**	Glutamine
R	**Arg**	Arginine
S	**Ser**	Serine
T	**Thr**	Threonine
V	**Val**	Valine
W	**Trp**	Tryptophan
Y	**Tyr**	Tyrosine
X	**Xxx**	undetermined or non-standard aa

Notations:

P-7L	amino acid residue P (proline) at position -7 of a given protein sequence changed to amino acid residue L (leucine)
G9R	amino acid residue G (glycine) at position 9 of a given protein sequence changed to amino acid residue R (arginine)
P^{89}	amino acid residue P (proline) at position 89 of a given protein sequence

I.

INTRODUCTION and OBJECTIVES

"The beginning of knowledge is the discovery of something we do not understand."

Frank Herbert (1920-1986)

I.1 Introduction

Powerful molecular biology tools are nowadays available that significantly help in fundamental research and much contribute to technical developments in many scientific domains. In particular, their impact in animal science has been considerable (Vignal *et al*., 2002). These techniques have applications so diverse as fraud control and animal products traceability (Botter *et al*., 2003; Brodman and Moor, 2003), genetic diversity characterization (Rendo *et al*., 2004) or parentage testing (Werner *et al*., 2004). It is also possible, through molecular techniques, to select animals having lower susceptibility to a disease (Brandsma *et al*., 2005), or favourable genotype for productive traits such as growth performance in cattle, sheep and pigs (Johnson *et al*., 2005; Maj *et al*., 2006; McRae *et al*., 2005; Taylor *et al*., 1998; Wimmers *et al*., 2002) or milk production and composition in dairy cattle (Blott *et al*., 2003; Kaminski *et al*., 2005; Shariflou *et al*., 2000) and sheep (Barillet *et al*., 2005; De Vries *et al*., 2005; Diez-Tascón *et al*., 2001).

The search for polymorphism in candidate genes thought to affect production traits has contributed to a better understanding of the basic biology of milk production and composition, and to intensify selection for these traits, namely in dairy cattle. Hence, effort has been made to identify candidate gene markers for milk selection within the somatotropic axis (Di Stasio *et al*., 2005; Parmentier *et al*., 1999; Renaville and Portetelle, 1998) with particular emphasis on growth hormone gene (*GH*) (Malveiro *et al*., 2001; Marques *et al*., 2003; Lagziel *et al*., 1999). Various studies have shown that administration of GH to lactating animal increases milk production and feed conversion efficiency without detrimental effects on milk composition.

The choice of new selection processes linked to the polymorphism at the GH, a hormone that plays an essential role in milk production, is a possibility for a faster genetic progress and thus flocks improvement since females can be tested at birth.

Some initial studies on molecular diversity of somatotropic axis' genes have been performed in the "Serra da Estrela" ovine breed (Barracosa, 1996; Ramos *et al*., 2002). The use of molecular markers to improve milk production could considerably contribute to speed up genetic progress in this autochthonous breed. Thus, it could have a major impact on the preservation of the breed as it should help in implementing more efficient breeding programs leading to increased productivity while maintaining flock size. Breeders would be stimulated to maintain autochthonous breeds instead of introducing foreign breeds characterised by higher milk productions but non-adapted to traditional Portuguese grazing conditions. The

preservation of native breed would thus be promoted and the biodiversity assured while providing financial conditions for maintaining farmers in the rural areas.

I.2 Objectives

Throughout the literature review of this work, the principal regulatory mechanisms of *GH* gene expression and their effects on milk production in lactating ruminants will be looked into. Special emphasis will be given to the important roles that GH, or other genes under its control, plays in the animal body growth until puberty, in mammary gland development during puberty, gestation and possibly in the early lactation period, and in the homeorhetic control of female metabolism during gestation and lactation. Some considerations will be made about the ethical questions linked to the use of exogenous GH or transgenic animals for milk production.

The main objectives of the present work were to identify and characterise nucleotidic polymorphisms naturally occurring at the *oGH* gene in "Serra da Estrela" sheep, and associate them with milk traits in order to evaluate GH as a useful candidate gene for marker assisted selection. To attain those objectives, both copies of the o*GH* gene were genotyped, putative transcription factors binding sites at each copy's promoter were screened and GH genotypes and phenotypes were established. Once the putative GH variants were identified, their associations with milk yield and composition were also evaluated.

II.

LITERATURE REVIEW

"Science is the knowledge of consequences and dependence of one fact upon another."

Thomas Hobbes (1588–1679)

II.1 Serra da Estrela ovine breed

The "Serra da Estrela" ovine breed was considered one of the best Portuguese dairy breeds in the 40s and one of the best in the world (Alberty cit. by Borrego, 1982). The official milk records were initiated in the years 1944/45. The Herd Book of the breed started in 1984 and is presently at ANCOSE (National Association of the Breeders of the "Serra da Estrela" Sheep). The totality of the milk yielded by "Serra da Estrela" ewes is transformed into "Serra da Estrela" cheese which has a Protected Denomination of Origin (PDO). This cheese is manufactured exclusively with milk from this ovine breed and is the main agricultural product of this region, and has a high socio-economical importance for rural populations.

Despite all the efforts towards genetic improvement of the "Serra da Estrela" breed, the milk yield levels have not increased (**Table II-1**) as in foreign breeds and the breed is nowadays considered a low milk yield breed in the Mediterranean context (Boyazoglu, 1991a cit by Georgoudis, 1998). Presently, the milk yield potential of the "Serra da Estrela" breed (148 l/150 d; Carolino *et al*., 2003b) is much lower than that of Awassi (506 l/214 d; Pollott and Gootwine, 2001, 2004), Assaf (334 l/173 d; Pollot and Gootwine, 2004) or Lacaune (270 l/165 d, Barillet *et al*., 2001; 230 l/145d at the 1st lactation, Rupp *et al*., 2003) breeds.

Table II-1. *Evolution of "Serra da Estrela" total milk yields (TMY; l) and milk yields in 150 days (MY; l/150 d) between 1944 and 2004.*

Years		n	Lactation lenght	TMY	MY	No. breeders	Reference
1944/1946	White	1989	220	100	nd	nd	Alberty, 1948 cit. by Borrego, 1982
	Black	466		135	nd		
1966/1985		6537	220	145.8	112.7	41	Delgado and Martin, 1992
1986/1990	Single	na	220	136.9	108.6	71	Gulbenkian, 1993
	Twin	na		143.1	114.4		
	Triple	na		144.6	118.9		
1993/1997	Single	51091	220	140.7	121.5	557	Carolino *et al*., 1997/1998
	Twin	32470		149.3	128.6		
1997/2004	Single	744	180.0	126.9	108.1	28*	ANCOSE, not published
	Twin	450	196.0	149.3	124.2		
	Triple	14	229.0	198.5	153.9		

n – number of lactations; na – non-available; nd – non-determined.
* 398 Artificial insemination born ewes (paternal grandmother with milk yield higher than 240 l/150 d in at least two lactations; Esteves, 1997/1998)

The factors pointed by Borrego (1982), i.e., the production system and the socio-economic-cultural structure of the shepherds, were responsible for the subsistence of traditional flock management practices, which in the end impaired the genetic improvement results of the

breed. Indeed, "Serra da Estrela" ovine breeders have seen no substantial improvement on flocks´ milk yields via the classical genetic selection programme. Simultaneously, ewes' milk prices have decreased in the Portuguese market (from € 1.00/l in 1995 to € 0.87/l in 2004; INE, 2006). The joint effect of these two factors could be the loss of economic sustainability of the traditional ovine production system based on "Serra da Estrela" ewes and the abandonment of this autochthonous breed in favour of higher yielding foreign breeds such as Lacaune, Assaf and Manchega. The number of "Serra da Estrela" ewes increased from 1999 to 2000 and has remained constant afterwards (**Figure II-1**). A possible way for the valorisation and genetic improvement of the breed is the inclusion of genetic markers in a breeding programme.

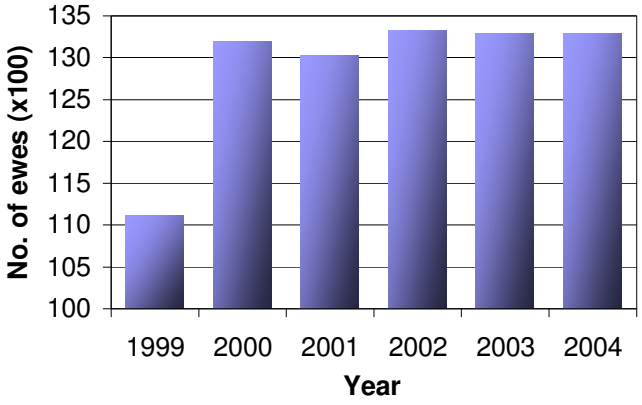

Figure II-1. *Evolution of the number of "Serra da Estrela" ewes inscribed in the breed Herd Book (FAO, 2004).*

The introduction of genetic markers in animal breeding programmes will allow a more rigorous selection, giving an increase of information about the genetic value of an animal. Animal identification early in life (in the embryo phase or immediately after parturition) will allow reducing the time interval between generations and will be useful for the selection of young females before the onset of their productive life, leading to the development of more efficient breeding programs. Several candidate genes can be proposed to be introduced into marker assisted selection (MAS), depending on the breeding programme objective. In "Serra da Estrela", the objectives are to improve milk yield and to minimize any negative effect on milk quality.

In ewes, results from Kann *et al.* (1999) suggest that mammogenesis and/or lactogenesis are partially controlled by somatotrophic hormones such as ovine growth hormone (o*GH*) and ovine placental lactogen (*PL*), and that insulin-like growth factor I (*IGF-I*) could be one of the mediators of these hormones. Therefore, the detection of genetic markers at the o*GH* gene associated with milk production and quality might contribute to the establishment of early selection criteria.

II.2 The growth hormone gene

The *GH* is a member of a multigene family which includes chorionic somatomammotropin, prolactin (PRL) and PL genes as well as several other genes, all of which evolved through series of gene duplications. Extensive reviews concerning the evolution, structure, function and molecular biology of the growth hormone gene family have been published (Bluet-Pajot *et al,* 1998; Cooke and Liebhaber, 1995; Chappel and Murphy, 2000; Forsyth and Wallis, 2002; Miller and Eberhardt, 1983). Thus, the present review will briefly focus on domestic animals' *GH*, with special emphasis on ovine (whenever information exists), its structure, regulation, metabolic effects and impact on animals' productive traits.

GH genes have been isolated and characterized in detail in different domestic animals species such as ovine (Byrne *et al.,* 1987; Guron *et al.,* 1992; Orian *et al*., 1988), caprine (Kioka *et al*., 1989; Yamano *et al*., 1988; Yato *et al*., 1988), bovine (Miller *et al*., 1980; Woychik *et al*., 1982), bubaline (Maithal *et al.,* 2001; Verma *et al.,* 1999), porcine (Chen *et al*., 1970; Vize and Wells, 1987), equine (Conde *et al*., 1973), dromedary (Martinat *et al*., 1990) and chicken (Zhvirblis *et al*., 1987).

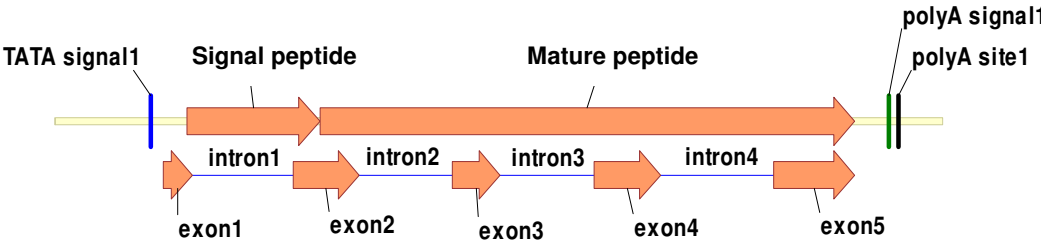

Figure II-2. *Schematic representation of oGH gene structure (2162 bp; Orian et al., 1988).*

The o*GH* gene has been mapped to 11q25 (Hediger *et al*., 1990) being entirely located within a 3.9 kb *Bam*HI/*Hind*III fragment (Byrne *et al*., 1987). The o*GH* coding sequence contains five exons with 71, 161, 117, 162 and 198 bp in length (according to Orian *et al*., 1988; see **Figure II-2**) interrupted by four introns (with sizes between 227 and 275 bp). Several highly conserved regions were described at the 5' end of the gene: a TATAAA sequence (TATA signal 1) is located at position -30 from the transcription starting point, and a CATAAAT sequence at position -84. The AATAAA polyadenylation signal (polyA signal 1 at position 2032) and polyA site1 (at position 2055) are present at the 3'-untranslated region (3'-UTR) of the gene.

The oGH predicted amino acid sequence, established by Orian *et al*. (1988) from an ovine pituitary genomic library, consists of a signal peptide composed of 26 amino acids in length and a mature peptide of 191 amino acids (Swiss-Prot accession no. P01247). The GH amino acid sequence is 99 % identical between ovine, caprine, bovine and bubaline, but differs markedly from non ruminant sequences [about 88% identical to pig GH (pGH) and 65% to human GH (hGH)].

In ovine, the *GH* family genes are expressed in the anterior pituitary somatotrophs (Sartin *et al*., 1996) and in the trophectoderm and syncytial placenta cells in a temporal-specific way (Lacroix *et al*., 1996; 1999).

II.2.1 o*GH* gene duplication

Two alleles of the *GH* gene have been described in ovine. The *Gh1* allele contains a single gene copy (*GH1*), whereas in the *Gh2* allele the gene is duplicated (*GH2-N* and *GH2-Z* copies) with the two copies being located 3.5 kb apart (Valinsky *et al*., 1990; **Figure II-3**). Individual animals homozygous for *Gh1* or *Gh2* alleles have two or four *GH*-like genes, respectively, while heterozygous animals with one allele of *Gh1* and one of *Gh2* (*Gh1*/*Gh2*), have three *GH*-like genes (Wallis *et al*., 1998). Sequence differences between the *GH2-N* and *GH2-Z* genes have been demonstrated and polymorphisms have been found in o*GH* coding and noncoding regions (Ofir and Gootwine, 1997).

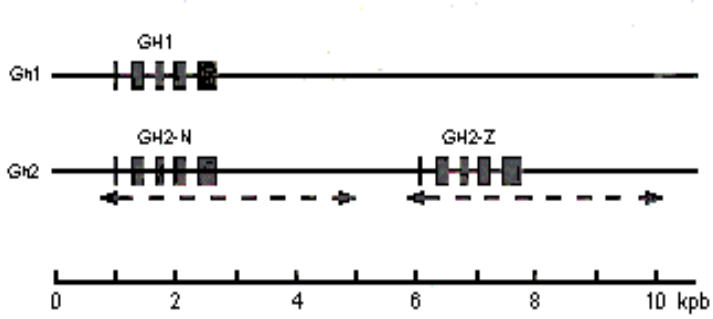

Figure II-3. *o*GH *gene alleles* Gh1 *and* Gh2 *in sheep (based on Valinsky* et al*., 1990).*
Exons are presented as black boxes. *GH1*, *GH2-N* and *GH2-Z* are the three copies of the o*GH* gene. Dashed arrows indicate the duplicated sequence (Adapted from Wallis *et al*., 1998).

A similar *GH* gene duplication has also been described in caprine (Yamano *et al*., 1991) but not in bovine, porcine or rats. In humans, the *GH* cluster comprises five *GH*-like genes in tandem sharing more than 95% sequence identity (Seeburg *et al*., 1982 cit. by Bennani-Bäiti *et al*., 1998). *GH* gene is also duplicated in some species of fish (Yang *et al*., 1997; Clements *et al*., 2004).

Sequence differences at the expressed protein level have been demonstrated. Lacroix *et al*. (1996) detected two GH-like proteins in the ovine placenta: one identical to the amino acid sequence deduced from the nucleotide sequence published by Orian *et al*. (1988) which is the pituitary product of the o*GH* gene (copy *GH1* or *GH2-N*); the other, is the product of the o*GH2-Z* copy gene, differs from the first in three amino acids: one at the signal peptide (P-7L), a second at the border of helix1 (G9R) of the GH molecule and a third one (G63S) at a loop structure of the binding site 1 (described in hGH; de Vos *et al*., 1992; see **II.4**).

II.3 Transcription regulation of the GH *gene*

Within the numerous transcription factors acting upon the anterior pituitary gland (Savage *et al*., 2003), several play a determinant role in somatotroph development and in the *GH* gene regulation. Binding sites to several of those transcription factors were disclosed by DNase I footprinting experiments, methylation-interference assays and band-shift analysis mainly at h*GH,* rat *GH* (r*GH*) and bovine *GH* (b*GH*). Putative binding sites could also be found at the o*GH* gene promoter (**Figure II-4**).

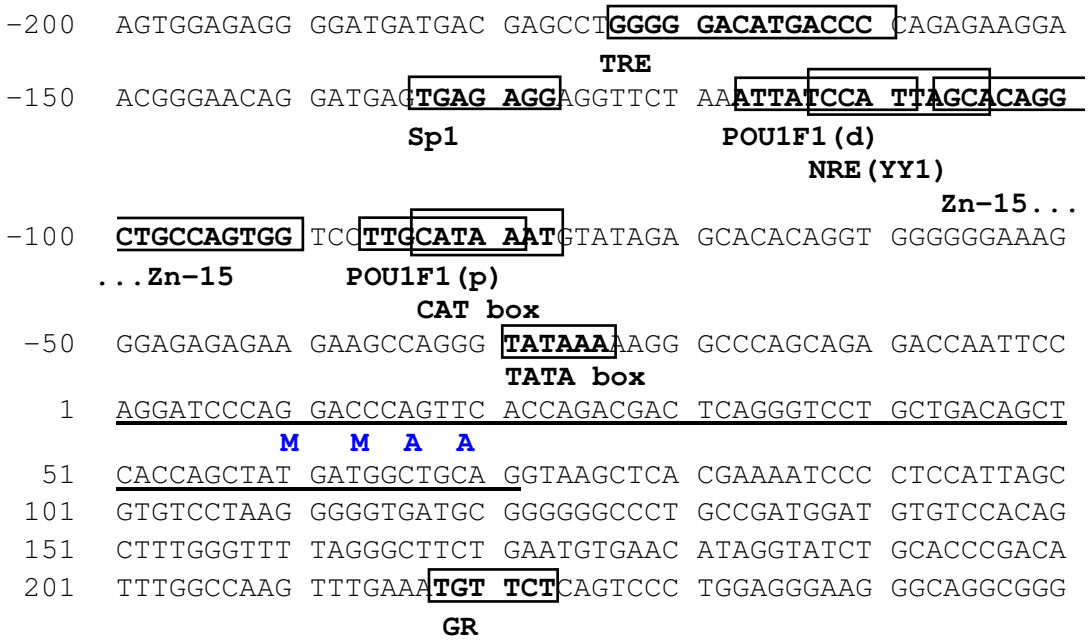

Figure II-4. *Regulatory sequences at the promoter of the o*GH *gene.*
The sequence of the sense strand of the *oGH* promotor region (from Orian *et al*., 1988) is shown together with the putative locations of the following transcription factors' binding sites (boxed bold sequences): TRE (T_3 response element), Sp1 (stimulating protein 1), POU1F1(d) and POU1F1(p) (distal and proximal pituitary-specific transcription factor 1), NRE (negative regulatory element), YY1 (yin and yang factor 1), Zn-15 (zinc finger protein) and GR (glucocorticoid receptor). CAT and TATA boxes are also shown. The exon 1 sequence is underlined, and the first four NH_2-terminal amino acids of the signal peptide are shown in blue.

Despite *GH* promoters' species-specific organization (Chuzhanova *et al*., 2000) throughout vertebrate evolution, some common features subsist. The following sub-sections will briefly focus on the general action of individual transcription factors (with special emphasis on *POU1F1*) and on the synergic action between them that lead to the transcriptional control of GH gene expression.

II.3.1 Pituitary-specific transcription factor (POU1F1)

Two pituitary-specific transcription factor (POU1F1, also called Pit-1, GHF-1, PUF-1 or GC-1) binding sites are present in the o*GH* promoter at position -118 (ATTATCCAT in agreement with the consensus sequences; Ingraham *et al*., 1988; Rhodes and Rosenfeld, 1996) and at position -87 [TTGCATAA; differs at 1 nt from hGH (Lemaigre *et al*., 1990) and at 2 nt from rGH (Kim *et al*., 1996)]. In addition to *GH* promoter POU1F1 binding sites, the presence of at least two POU1F1 binding sites within the chromatin Dnase I hypersensitive sites (HS I, II – F14 segment) of the locus control region (LCR; Jones *et al*., 1995) located -14.5 kb upstream of the h*GH-N* gene seem to be fundamental for the appropriate pituitary h*GH-N* gene expression in transgenic mouse (Shewchuk *et al*., 1999). Whether this LCR is also important for *GH* expression in transgenic sheep with additional *GH* copies was not discussed (Adams *et al*., 2002, 2005).

POU1F1 is a pituitary-specific POU-homeodomain protein (Ingraham *et al*., 1988) essential for thyrotrophs, somatotrophs and lactotrophs differentiation and survival (Li *et al*., 1990). It regulates the *GH* gene expression activation at the somatotrophs and its repression at the lactotrophs (Scully *et al*., 2000).

In the early embryonic life following the formation of Rathke's pouch, the primordium of the pituitary gland, *POU1F1* expression is activated by the Prophet of POU1F1 (PROP1) binding to *POU1F1* early enhancer (Sornson *et al*., 1996), possibly not alone but with the synergic action of vitamin D receptor and retinoic acid receptor (RAR) binding (Cohen *et al*., 1999; DiMattia *et al*., 1997). As soon as levels of PROP1 decay, *POU1F1* expression changes to an auto-regulatory pathway (Rhodes *et al*., 1993 cit by Sornson *et al*., 1996).

POU1F1 expression is dependent on its auto-regulation as *POU1F1* promoter contains several binding sites to POU1F1 at its proximal and distal regions (Rhodes *et al.,* 1993 cit by DiMattia *et al*., 1997) and is age (DiMattia *et al*., 1997) and gender (Gonzalez-Parra *et al*., 1996) dependent. Distinct signal-transduction pathways could thus regulate POU1F1 activity. These pathways are mediated by intracellular levels of cyclic adenosine 3',5'-monophosphate

(cAMP) or by mitogen-activated protein kinases (MAPK) and/or protein kinase A (PKA) activities in response to the epidermal growth factor (EGF) or insulin and involve a co-repressor complex containing the nuclear receptor co-repressor N-CoR/SMRT, mSin3A/B and histone deacetylases, and a co-activator complex containing cAMP-response element binding protein (CREB) and p/CAF (Xu *et al*., 1998).

POU1F1 activity depends also on other peptides, e.g. activin inhibits POU1F1 binding to *GH* promoter and consequently *GH* expression (Struthers *et al*., 1992). This effect is mediated by an increase in POU1F1 phosphorylation which also decreases POU1F1 stability (Gaddy-Kurten and Vale, 1995).

Mutations at the *POU1F1* gene interrupt the normal development of the anterior pituitary gland (dwarf genotype; Li *et al*., 1990; Pfäffle *et al*., 1992; 1993; Aarskog *et al*., 1997) and may lead to combined pituitary hormone deficiency (Cohen *et al*., 1995; Vallette-Kasic *et al*., 2001). *POU1F1* genotypes affect milk yield in humans (Pfäffle *et al*., 1996), milk yield and conformational traits in bovine (Renaville *et al*., 1997), and plasma GH and PRL levels (Sun *et al*., 2002) and growth and carcass traits in porcine (Stancekova *et al*., 1999; Yu *et al*., 1995). However, in other studies no associations were found between productive traits and *POU1F1* genotypes, e.g., with meat production traits in Piemontese bovine breed (Di Stasio *et al*., 2002).

II.3.2 Thyroid hormone and retinoic acid receptors response elements

One 3,5,3'-triiodothyronine receptor (T3R) response element (TRE)/ retinoic acid receptor (RAR) element is present in the o*GH* promoter at position -172 (GGGACATGACCC identical to b*GH*; Brent *et al*., 1988 cit. by Williams *et al*., 1992). The presence of such an element near the POU1F1 binding site is concomitant with the finding in rat, that *GH* transcription is enhanced by straight cooperation between T3R and RAR and POU1F1 via direct protein-protein interaction (Palomino *et al*., 1998).

II.3.3 Glucocorticoid receptor (GR)

A glucocorticoid receptor (GR) site is present in the o*GH* promoter at position +218 (TGTTCT) within the intron 1 as in the h*GH* (Moore *et al*., 1985) and in the goat *GH* (g*GH*; Kioka *et al*., 1989) promoters. Several works conducted in rat (reviewed by Theill and Karin, 1993) and in humans (Isaacs *et al*., 1987 cit. by Theill and Karin, 1993) reported that the *GH* gene expression was stimulated by glucocorticoids (GCs) and that GCs promote h*GH* mRNA stability (Paek and Axel, 1987 cit. by Theill and Karin, 1993). However, familial GC

deficiency has been associated with tall stature in human (Elias *et al*., 2000 cit. by van der Eerden *et al*., 2003), which is in accordance with findings that GC inhibits GH release in pituitary (Allen, 1996 and Luo and Murphy**,** 1989 cited by van der Eerden *et al*., 2003). Thus, considering the conflicting results obtained in the referred two species and that regulation of *GH* transcription by GR was not investigated in ovine, the real impact of GCs on o*GH* gene remains to be clarified.

II.3.4 Ubiquitous transcription factors

II.3.4.1 Stimulating protein 1 (Sp1)

One stimulating protein 1 (Sp1) binding site is present in the o*GH* promoter at position -134 [TGAGAGG; different in 1 nt from r*GH* (Kim *et al*., 1996)]. This site is near to the POU1F1 distal binding site and some authors suggested that Sp1 and POU1F1 binding could be mutually exclusive (Lemaigre *et al*., 1990). Nevertheless, Sp1 is thought to positively influence *GH* expression.

II.3.4.2 Zinc finger protein (Zn15/Zn16)

A zinc finger protein (Zn15/Zn16) binding site is present in the o*GH* promoter at position -108 (AGCACAGGCTGCCAGTGG; Lipkin *et al*., 1993 cit by Das *et al*., 1996). Zn15/Zn16 is a member of the Cys/His zinc finger transcription factor superfamily which can act synergistically with POU1F1 to enhance *GH* expression (VanderHeyden *et al*., 2000).

II.3.5 Silencer element

A negative regulatory element (NRE; sequence TCCATTAGC at position -114) with sequence similar to the yin and yang factor 1 (YY1) binding site, described in bovine (Park and Roe, 1996a, 1996b) and red deer (Lioupis *et al*., 1997), is present in the o*GH* promoter. Park and Roe's results (1996b) suggest that b*GH* expression is negatively regulated by YY1 or by a very similar YY1 homolog via NRE binding.

The joint action of the tissue-specific POU1F1 trans-acting factor and the ubiquitous factors referred before (and possibly others) should contribute to the tissue specific transcription of o*GH* gene, as it has been observed in other species.

II.4 Neuroendocrine regulation of GH secretion

Regulatory pathways of GH expression are not straightforward. They involve chain reactions whereby a peptide could simultaneously regulate expression of several genes. Transcription regulation of *GH* gene was addressed in the previous section, but many of those transcription factors are actively involved in the regulation of other peptides which in turn regulate GH expression. An example is POU1F1: it regulates GH-releasing hormone receptor (GHRHR) gene expression, and thus indirectly GH secretion by GH-releasing hormone (GHRH) signalling (Godfrey *et al*., 1993 cit by DiMattia *et al*., 1997).

Pituitary somatotroph cells possess receptors to GHRH, somatostatin (somatotropin release-inhibiting factor, SRIF), pituitary adenylate cyclase-activating polypeptide (PACAP) and GH secretagogue (ghrelin) peptides that control the GH expression at those cells.

Hypothalamic neurons secreting GHRH are located in the arcuate nucleus and those secreting SRIF are in the periventricular nucleus and arcuate nucleus (Leshin *et al*., 1994 cit by McMahon *et al*., 2000). GHRH and SRIF could mutually inhibit each other's synthesis in the arcuate nucleus neurons, but GHRH and SRIF syntheses and consequently GH regulation could be modulated also by neuropeptides such as leptin, insulin and IGF-1, dopamine, norepinephrine, serotonin, thyrotropin-releasing hormone, acetylcholine, neuropeptide Y (NPY) and galanin (see McMahon *et al*., 2001). Their influence upon GH synthesis and secretion of some of those peptides will be briefly discussed.

II.4.1 Growth hormone-releasing hormone (GHRH)

GHRH is structurally related to the glucagon superfamily, which include also PACAP, glucagons and glucose-dependent insulin-inducing peptide (GIP) (Cummings *et al*., 2002; for review see Mayo *et al*., 2003).

GHRH stimulates equally the synthesis and the GH pulse secretion in sheep. Indeed, it was observed a significant association between GHRH and GH peaks, but not between SRIF and GH peaks in unanesthetized ovariectomized ewes (Frohman *et al*., 1990). Moreover, other studies reported an increase in plasma GH levels when lambs were injected with a GHRH analogue with increased feed conversion and leaner carcasses (Godfredson *et al*., 1990), and recently the injection of a myogenic expression GHRH plasmid DNA into Inner Mongolia fuzz lambs resulted in higher GH levels and in organomegaly (Meng *et al*., 2004).

The GHRH-stimulated GH release in the somatotrophs is presumably cAMP- and Ca^{2+}-dependent in sheep (Sartin *et al*., 1996). *In vitro* results suggest that Ca^{2+} pathway presumably acts via calmodulin activation and concomitant/subsequent activation of PKA which promotes GH release induced by cAMP (Sartin *et al*., 1996; for a review see Bluet-Pajot *et al*., 1998).

II.4.2 Somatostatin (SRIF)

SRIF is a hypothalamic cyclic polypeptide with two bioactive isoforms (SRIF-14 and SRIF-28; Møller *et al*., 2003) that negatively regulate GH release (Davis, 1975; Kazmer *et al*., 2000) by SRIF receptors (sst-1 and sst-2) activation. Besides GH, an extensive list of hormones is inhibited by SRIF; among them are PRL, insulin, thyroid-stimulating hormone and almost all hormones from the gastrointestinal tract, inhibiting also the nutrient absorption at intestinal level (reviewed by Møller *et al*., 2003).

SRIF binds to its receptor activating the cAMP and phosphoinositide signal transduction pathways (Møller *et al*., 2003). Specifically and in opposition to what happens when GHRH binds to its receptor, when SRIF binds to guanyl nucleotide-binding proteins (G_I-proteins) linked cell surface receptor, the activity of the adenylate cyclase is reduced resulting in lower levels of cAMP and in PKA inhibition. PKA inhibition reduces CREB phosphorylation, a key step in *POU1F1* transcription regulation. SRIH and GHRH signal transduction pathways converge at Ca^{2+} ion channels with presumably metabolic antagonist effect.

Under insulin hypoglycaemia conditions, SRIF secretion increases and in response, GH levels decrease in ewes (Frohaman *et al*., 1990). However, when lactating ewes were immunized against SRIF (Sun *et al*., 1990), no GH level change was observed yet milk yield increased. It was suggested that more nutrients reach the mammary gland in response to the blocking of the inhibitory action of SRIF upon the gastrointestinal tract and consequent increase in nutrient absorption. In growing cattle also immunized against SRIF, Ingvartsen and Sejrsen (1995 cit. by Ingvartsen and Andersen, 2000) observed a somehow similar effect as the animals grew faster and, despite a higher feed intake, the feed conversion ratio improved.

II.4.3 Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP is a neuropeptide expressed in the central nervous system acting simultaneously as a neurohormone and a neurotransmitter (Montero *et al*., 2000). It is also

expressed within the gonads (reviewed by Moretti *et al*., 2002) and the adrenal gland (Ghatei *et al*., 1993 cit by Cummings *et al*., 2002). In ovine, PACAP was found to stimulate the accumulation of cAMP in the pituitary cells (Miyata *et al*., 1989 and 1990 cit. by Cummings *et al*., 2002) similarly to what occurs after GHRH stimulation. Moreover, in meal-fed Holstein steers injected with PACAP before feeding, the GH peak levels increased in serum. It was thus postulated that PACAP induces GH secretion maybe in association with GHRH (Radcliff *et al*., 2001).

II.4.4 Ghrelin

Ghrelin (also known as GH secretagogue) is a growth hormone-releasing acylated peptide synthesised in the oxyntic glands of the stomach and intestine (Date *et al*., 2000). It stimulates GH secretion in small ruminants (Hayashida *et al*., 2001; Sugino *et al*., 2004) by activating ghrelin receptor (reviewed by Davenport *et al*., 2005) in the pituitary. It is thought that ghrelin may have a role in feeding regulation in domestic animals and thus in energy homeostasis, as it was suggested by the results of studies in ghrelin knockout mice (Wortley *et al*., 2005) and in fat and lean lines of sheep (French *et al*., 2006).

Ghrelin and GHRH act synergistically to promote GH release. Moreover, GHRH and GH-releasing peptide 2 (GHRP-2; a synthetic secretagogue) were shown to regulate their receptor synthesis in a time-dependent way in ovine pituitary cell cultures, playing also an important role in *GH*, *GHRHR*, *POU1F1*, *sst-1* and *sst-2* expression and GH synthesis (Yan *et al*., 2004). Ghrelin regulation of GH secretion could also be mediated by the activation of the NPY-Y1 receptor pathway (Shintani *et al*., 2001).

The first studies with synthetic secretagogues (GHRP-6 and more recently GHRP-2) showed that secretagogues enhance GH secretion by a different via than that used by GHRH. GH-release via ghrelin involves inhibition of K^+ channels and somatotroph depolarization with consequent Ca^{2+} channel rearrangement. However, GHRH and ghrelin pathways communicate trough signalling pathways involving intricate ionic exchanges (Chen *et al*., 1996 cit. by Casanueva *et al*., 1999).

II.4.5 Leptin

Leptin (or OB protein) is the product of the *ob* gene expression in the adipose tissue (Zhang *et al*., 1994 cit. by Schwartz *et al*., 1996) and placenta (Anthony *et al*., 2001). The leptin levels are positively correlated with body fat mass (Delavaud *et al*., 2002), energy intake

level (Marie *et al*., 2001; Reist *et al*., 2003), β-adrenergic stimulation (Chilliard *et al*., 2000) and negatively correlated with photoperiod in sheep (Marie *et al*., 2001; Clarke *et al*., 2003). Leptin receptors are expressed in ewe hypothalamus, anterior pituitary and adipose tissues (Dyer *et al*., 1997c), and mammary gland (Laud *et al*., 1999), and in bovine adrenal medullary cells (Yanagihara *et al*., 2000). Main effects of leptin were reviewed by Chilliard *et al*. (2001), Ingvartsen and Boisclair (2001) and Faggioni *et al*. (2001).

A high level of leptin, which could be observed after a meal or in obese animals, was shown to physiologically regulate food intake by decreasing appetite (Barb *et al*., 1998). At the same time, high levels of leptin increase energy demands and, to meet those demands, there is an increased fatty acid oxidation, i.e., lipolysis at the adipose tissue, or alternatively a decrease in insulin-stimulated lipogenesis (Ramsay, 2001). However, in other studies this effect was not observed (Newby *et al*., 2001). Those metabolic events are mediated by modification at hormonal levels, namely GH, catecholamines and T_3 augment, and insulin and GCs decline (see review by Chilliard *et al*., 2001).

Leptin acts upon the expression of hypothalamic neuropeptides involved in energy homeostasis. It was observed that after leptin injection NPY expression decreased in the arcuate nucleus and corticotrophin releasing hormone increased in the paraventricular nucleus in normal Long-Evans rats (Schwartz *et al*., 1996). This could cause GH levels to decrease which mimic the observed decrease of the GH levels after meals in sheep (Matsunaga *et al*., 1999) or, following another via, could decrease GHRH-stimulated *GH* expression and at the same time increase basal GH as it was observed *in vitro* in ovine pituitary cells (Roh *et al*., 1998) and plasmatic GH *in vivo* without altered GH pulse frequency (Nagatani *et al*., 2000).

Leptin regulates short- and long-term homeostasis, thus some of the opposite effects observed concerning *GH* expression regulation could reflect complex feedback mechanisms that support the return to a steady levels of body fat after episodes of mobilization/deposition of fat tissue.

II.4.6 Other GH-Regulating Neuropeptides

II.4.6.1 Catecholamines

Two catecholamines are particularly important in GH regulation in domestic animals: dopamine and norepinephrine.

Dopamine has been found to inhibit GH secretion via D_1- and D_2-like dopamine receptors. Stimulation of dopamine D_1-receptors and subsequent enhanced activity of

periventricular SRIF neurons increases SRIF secretion into hypophysial-portal vessels and GHRH-induced release of GH into blood decreases in steers (McMahon *et al*., 1998; West *et al*., 1997). In addition, dopamine or D_2-receptors activation inhibit GHRH-induced GH secretion and decrease cAMP levels in sheep pituitary cells culture (Law *et al*., 1984).

Norepinephrine effects are mediated by its α_1-, α_2- and β-adrenergic receptors. It was observed that GH secretion is inhibited by α_1- and stimulated by α_2-adrenergic receptors activation in rat (Willoughby *et al*., 1993). α_2-adrenergic receptor activation also stimulated GH secretion in ovine (Soyoola *et al*., 1994 cit. by McMahon *et al*., 2001) and bovine (Gaynor *et al*., 1993). Furthermore, pulse GH secretion before feeding and basal GH levels post-feeding are apparently mediated by α_2-adrenergic receptor stimulation in Holstein steers (Gaynor *et al*., 1993). β-adrenergic receptors activation was found to inhibit GH secretion hypothetically by SRIF-enhanced secretion in ewes (Thomas *et al*., 1994).

II.4.6.2 Neuropeptide Y (NPY)

NPY is an orexigenic peptide from the pancreatic peptide family. In ovine, the expression of the NPY-Y1 receptor was seen within the arcuate nucleus and paraventricular nucleus of the hypothalamus, the dentate gyrus of the hippocampus and in pancreatic, anterior pituitary, and adipose tissues, and the expression of the NPY-Y2 receptor within hippocampus and within pancreatic tissue (Dyer *et al*., 1997a).

Administration of NPY was shown to strongly increase GH secretion in sheep (Morrison *et al*., 2003) and cattle (Garcia *et al*., 2004; Thomas *et al*., 1999). However, the effect of NPY on GH secretion was attenuated if cows were primarily injected with leptin (Garcia *et al*., 2004). Furthermore, in a comparative study, underfed ewes presented higher number of immunoreactive cells for NPY at the arcuate nucleus and median eminence, higher density of NPY terminal fields at arcuate nucleus and pre-optic area, paraventricular nucleus, and higher plasma levels of GH than well-fed ewes (Barker-Gibb and Clarke, 1996). Taken together, those observations suggest that NPY is a mediator signal reporting overall body energy status to the brain, probably within an insulin-leptin-NPY pathway as hypothesised by Dyer *et al*. (1997b), where ghrelin could also be involved (Shintani *et al*., 2001).

II.4.6.3 Galanin

Galanin neurons were found in several regions of the hypothalamus. Galanin has two subtypes of receptors: GalR1 and GalR2 (Wang *et al*., 1998 cit. by McMahon *et al*., 2001). In pituitary slices of young male calves, galanin was shown to have a significant stimulatory effect

upon GH secretion (Baratta *et al*., 1997). This effect was also found in sheep (Saleri *et al*., 1999), humans and rat (see Giustina and Veldhuis, 1998).

Immunization against GHRH inhibits the GH response to galanin, suggesting a galanin-GHRH interaction (Murakami *et al*., 1989 cit by Giustina and Veldhuis, 1998).

II.4.6.4 Neurotransmiters amino acids

Amino acids influence polarization status of central nervous system cells by exciting (depolarizing) or inhibiting (hyperpolarizing) the cells. Cells could be excited by aspartic acid, cysteic acid, glutamic acid, and homocysteic acid, and inhibited by γ-aminobutyric acid (GABA), glycine, taurine, and β-alanine, with consequences on GH secretion regulation (Cooper *et al*., 1996 cit by McMahon *et al*., 2001; Müller *et al*., 1999).

For instance, intravenous administration of GABA resulted in a rapid and significant increase in plasma GH, however intracerebroventricular administration of GABA changed plasma GH levels in a dose dependent manner: 10 mg of GABA increased and 100 mg decreased plasma GH levels. Results also point out to the possible existence of a mechanism independent from GHRH/SRIF, which could mediate GABA regulating effect upon GH secretion in sheep (Spencer *et al*., 1994).

II.5 GH signal transduction regulation

The three dimensional structures of pig (Abdel-Meguid *et al*., 1987), bovine (Carlacci *et al*., 1991) and human (Cunnigham *et al*., 1991; de Vos *et al*., 1992) GH proteins have been described. The oGH, as other members of the family of hematopoietic cytokines, is expected to comprise an anti-parallel twisted four-α-helix bundle with "up-up-down-down" topology, with two long loops, linking helices 1 and 2, and 3 and 4 respectively, and a short segment connecting helices 2 and 3 (Abdel-Meguid *et al*., 1987; Wells and de Vos, 1993). Structure-function results obtained for pGH and hGH have no direct application to oGH, nevertheless some assumptions can be made. In analogy with pGH, the four helices of the oGH are localised between residues 7-34, 75-96 (kinked at P^{89}), 107-128 and 153-183, respectively (**Figure II-5**). In the hGH three short helical segments were also described: two

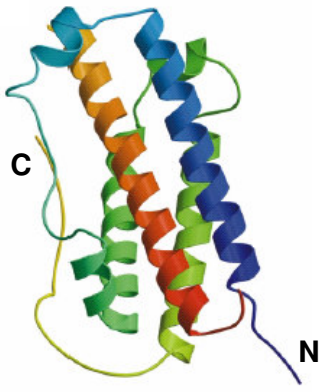

Figure II-5. *Rainbow-colored ribbon diagram of the hGH from the 3HHR crystallographic structure.*
Adapted from Keeler *et al*. (2003).
C – COOH-terminus;
N – NH_2-terminus.

of them between residues K^{38}-N^{47} and R^{64}-K^{70}, within the first long crossover connection, were involved in hormone-receptor contacts; and the third one between residues R^{94}-S^{100} located between helices 2 and 3 (de Vos *et al*., 1992).

The pGH presents two disulfide bridges connecting C^{53} in the first crossover connection to C^{164} in helix 4, and C^{181} in helix 4 to C^{189} near the COOH-terminus (Abdel-Meguid *et al*., 1987). These connections were also described in hGH (de Vos *et al*., 1992) along with several hydrogen-bonds which contribute to the four helix-bundle at GH hydrophobic core.

II.5.1 GH binding to cellular GH receptors

The biological effects of the GH are mediated by numerous second messenger pathways, activated in response to GH binding to specific cell surface GH receptors (GHR; Allan *et al*., 1999). In sheep the GHR was characterized by Adam *et al*. (1990).

The signal transduction of hGH is only achieved by the formation of the ternary complex between hGH and its receptor's extracellular domain (hGHBP) (X-ray crystal structure described by de Vos *et al*., 1992; **Figure II-6**) where a single hGH molecule binds sequentially to two hGHBP molecules (Cunningham *et al*., 1991; Wells, 1996), i.e., firstly hGH binds site 1 to a hGHBP, and then, binds site 2 to a second hGHBP. GH has also the ability to bind functionally to PRL receptors (PRLR; Barash *et al*., 1988; Cunningham and Wells, 1991; Kossiakoff *et al*., 1994; Somers *et al*., 1994), thus presenting lactogenic activity in addition to the somatogenic activity.

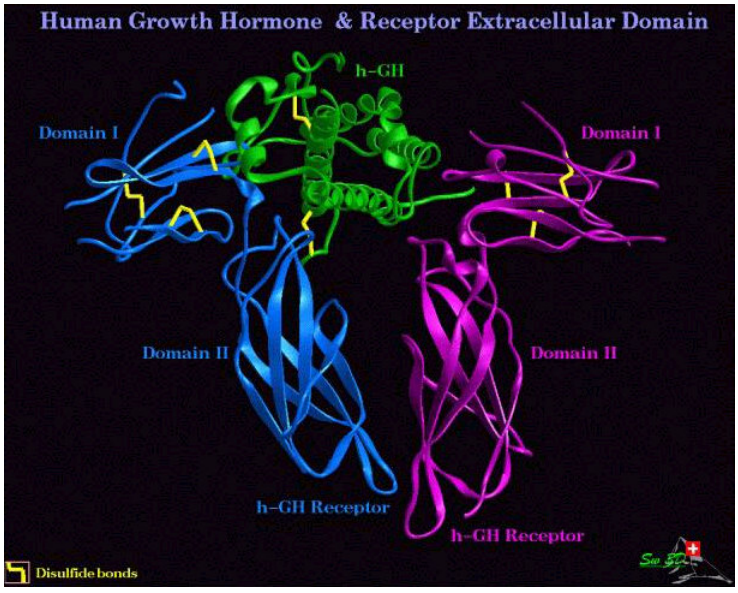

Figure II-6. *The structure of the 1:2 complex of the hGH with the hGHR extracelular domain (hGH/$hGHBP_2$ complex).*
Adapted from www.expassy.org.

The hGH amino acid residues involved in hGH/hGHBP binding sites 1 and 2 have been elucidated by Cunningham *et al*. (1991) and de Vos *et al*. (1992): site 1 - residues of the helix 1, of the short helical segment connecting helices 1 and 2 and more than half of the residues of the helix 4; site 2 - 13 residues across NH_2-terminus, the first residues of helix 1 and some residues of helix 3. In a subsequent review of the structure and function of the hGH (Wells and de Vos, 1993), the authors reported that only part of the hGH residues involved in hGH/hGHBP contact interface are actually functional epitopes, i.e., residues whose substitution generate a twofold reduction in binding activity. It has also been emphasised the hypothesis that many of the hydrophilic contacts are functionally silent or deleterious. Recently, Kouadio *et al*. (2005) showed by shotgun alanine scanning that minimal binding sites are necessary for functional hGH binding to its receptor. The authors also demonstrated that the stability of the ternary hGH/$hGHBP_2$ complex depends on the hormone – receptor affinity for binding site 2.

The extracellular domain of the hGHR covers two partial domains (**Figure II-6**): the NH_2-terminal domain I and the COOH-terminal domain II β-sheets. From the crystallographic structure of the hGH/$hGHBP_2$ complex, it is clear that the site 1 at hGH is larger than site 2, and that hGHBPs' binding residues are mostly the same, i.e. S^{145}, H^{150}, D^{152}, Y^{200} and S^{201}, showing also, similar structures (de Vos *et al*., 1992).

GH mutations might prevent receptor dimerization [e.g. hGH(G120R); Ultsch and de Vos, 1993; Clackson *et al*., 1998] and cause diseases and/or growth disorders such as short stature in humans by bio-inactivation of GH (Takahashi *et al*., 1997). However, mutations have been described that do no block GH/GHR binding activity, e.g. the ovine GH molecule with deletion of residues 33 through 46 continues to show significant GHR-binding activity (Sami *et al*., 1999). Another GH mutant molecule such as the hGH^{44-191} has been reported to retain high affinity to lactogenic receptors (PRLR) but not to the somatogenic ones (GHR) (Haro *et al*., 1996).

II.5.2 GH-dependent activation of JAK2

When GH binds to cell surface, it induces GHR homodimerization and consequent conformational changes that trigger GHR association with the intracellular Janus tyrosine kinase 2 (JAK2; Argetsinger *et al*., 1993), which phosphorilates both GHR and JAK2 itself, creating high-affinity binding sites for several signalling molecules with Src homology 2 (SH2) and phosphotyrosine binding sites (Carter-Su *et al*., 2000). The tyrosine residues of the GHR necessary for association and activation of the JAK2 were identified in Chinese hamster ovary

cells expressing wild-type or truncated GHR (VanderKuur *et al*., 1994) and also in humans (Hansen *et al*., 1996) and pigs (Wang *et al*., 1996) by GHR mutational studies (**Figure II-7**). Activation of JAK2 is the initial event for GHR signal, mediating several biological responses in the cells, e.g.: stimulation of tyrosyl phosphorylation of p97, activation of MAPK, protein kinase C (PKC) and signal transducer and activator of transcription (STAT) pathways and *Spi2.1* and *c-fos* expression, increase of protein synthesis and insulin secretion, and thus influencing metabolism and cellular proliferation and differentiation (reviewed by Carter-Su *et al*., 2000).

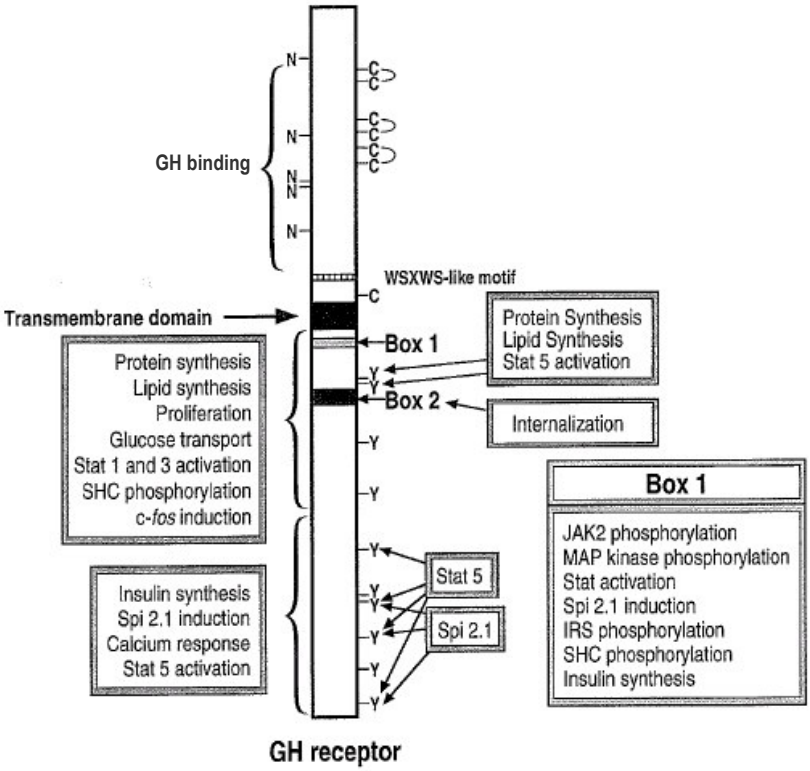

Figure II-7. *Binding sites at the GHR responsive for GH binding and GH-GHR signal transduction and their putative biological functions.*
C – extracellular cysteines linked by disulfide bonds; N – potential N-linked glycosylation sites; Y – tyrosines of rat GHR cytoplasmic region; Box 1 and Box 2 – intracellular proline rich domains; IRS – insulin receptor substrate; MAP kinase – mitogen-activated protein kinase; SHC – Src homologous containing proteins; Spi2.1 – serine protease inhibitor 2.1; STAT – signal transducer and activator of transcription. Adapted from Argetsinger and Carter-Su (1996) and Carter-Su *et al*. (1996).

II.5.3 Activation of MAPK signalling pathway

JAK2 activation could be the trigger event for the activation of other tyrosine kinases or for the inhibition of tyrosine phosphorylases. One of the pathways affected by such stimuli involves MAPK and extracellular-signal-regulated kinases 1 and 2 (ERK1/2) proteins which are phosphorylated in reply to GH stimuli, mediating pathways controlling cellular growth,

differentiation (Cobb and Goldsmith, 1995 cit. by Argetsinger and Carter-Su, 1996) and Ca^{2+} equilibrium (Olszewska-Pazdrak *et al*., 2004 cit. by Werry *et al*., 2005).

The Ras-MAPK-dependent membrane receptor tyrosine kinase activation is another via of GHR signalling mediated by Src homologous containing (Shc), Grb2, son-of-sevenless (Sos), ras, raf, and also by MAP/ERK kinase (MEK) (Smit *et al*., 1999 cit. by Carter-Su *et al*., 2000). Additionally, MAPK is a mediator between seven-transmembrane spanning (G-protein-coupled) receptors and their target effectors ERK1/2, in processes involving feedback regulation of the phospholipase A2, phosphodiesterases and cytoskeletal proteins and also downregulating MAPK phosphatase 1 (MKP1) (Werry *et al*., 2005).

II.5.4 GH signalling through PKC

The ability of GH to regulate several metabolic pathways is reduced by the inhibition of PKC (Argetsinger and Carter-Su, 1996). There are two second messengers that mediate PKC activation and translocation to the cytosol: Ca^{2+} and 1,2-diacylglycerol (DAG). Distinct pathways are thought to generate those messengers after GH stimulation depending on cell type: the inositol 1,4,5-triphosphate pathway generates Ca^{2+} and DAG; alternatively, in another pathway phospholipase C or D are necessary to hydrolyse the phosphatidylcholine generating only DAG (Argetsinger and Carter-Su, 1996).

II.5.5 GH-dependent activation of the STAT proteins family

Various signal transducers and activators of transcription (STATs) were identified until now (STAT1, 2, 3, 4, 5a, 5b and 6; Wakao *et al*., 1992, 1994; Liu *et al*., 1995; Silva *et al*., 1996), all of them containing a SH2 domain. Many of the functional roles of STAT family proteins were understood after studies in knockout mice (see Akira *et al*., 1999).

STATs play an important role in the early embryogenesis, and in the GH-regulated somatic growth pathway, by enhancing insulin (Galsgaard *et al*., 1996), *IGF-I* (Wang and Jiang, 2005), and acid-labile subunit (*ALS*) of the IGF-binding protein-3 complex (*IGFBP-3*) expression in the liver (namely through STAT5b; Woelfle and Rotwein, 2004). Furthermore, STAT5 (also known as mammary gland factor, MGF) identified in sheep mammary gland (Wakao *et al*., 1994), is involved in the milk secretion by regulating the mammary gland development (Liu *et al*., 1995; Matsumoto *et al*., 1999; Iavnilovitch et al., 2002), and the transcription of milk caseins (Inuzuka *et al*., 1999; Schmitt-Ney *et al*., 1991; Wakao *et al*.,

1992, 1994; Wartmann *et al*., 1996) and whey acidic proteins (WAP; Li and Rosen, 1995; Jura *et al*., 2005; Mukhopadhyay *et al*., 2001).

STATs, activated after GH-induced tyrosine phosphorylation (Xu *et al*., 1996), could form homodimers or heterodimers that, binding to specific sites at different genes, enhance the expression of those genes, e.g.: STAT5 homodimer binds to *IGF-I* intron 2 at the HS7 site (Woelfle and Rotwein, 2004), enhancing IGF-1 expression by interacting with the transcription factors present at the two *IGF-I* promoters (**Figure II-8**). The GH-STAT-IGF-1 axis is the main system of GH-transduction signalling in living organisms, mediating many of the GH biological function.

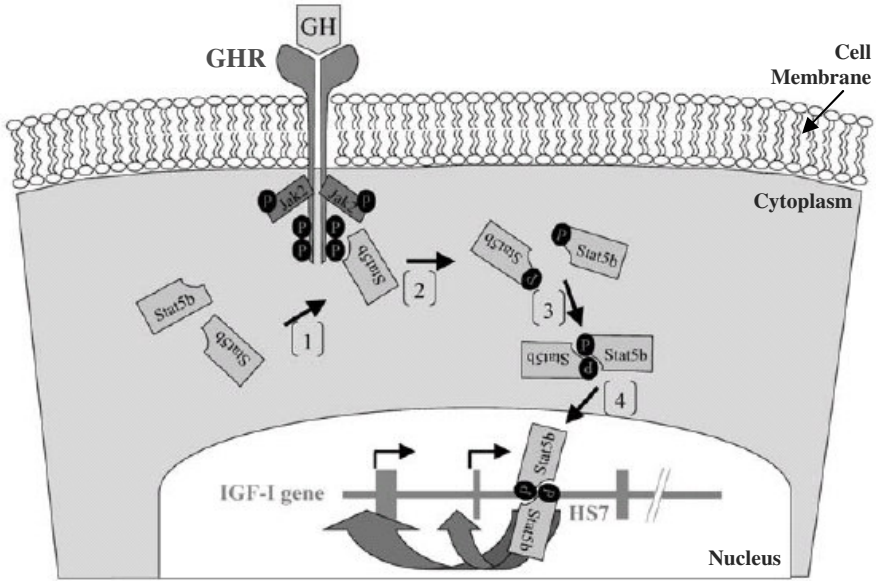

Figure II-8. *Diagram of the GH-dependent transcription of the insulin-like growth factor I* (IGF-I) *gene pathway.*
GH-GHR binding induces GHR dimerization and association, and activation of the JAK2 tyrosine kinase in the cell cytoplasm, with GHR-JAK2 cross-phosphorylation (1). STAT5b is tyrosine phosphorylated at the GHR (2) and form homodimers (3) which migrate to the cell nucleus (4). Within the nucleus, STAT5b binds to *IGF-I* intron 2 at the HS7 site, and interacting with the transcription factors present at the two *IGF-I* promoters (large arrows), it induces IGF-I gene transcription (angled arrows). Adapted from Woelfle *et al*. (2005).

II.5.6 GH signalling inhibition

GH secretion could be regulated by GH-dependent and/or independent negative feedback mechanisms. High concentration of GH can negatively regulate GH signalling by saturating GHR, inducing a dose dependent response to GH somehow similar to the response obtained when GH-antagonists are used (Frank, 2002; **Figure II-9**). It is thought that, as GHR has high affinity for the GH binding site 1, and because binding to sites 1 and 2 occurs sequentially (see **section I.4.1**), low number of GHR will be available for the formation of the

GH/GHR_2 ternary complex, fundamental for the correct GH-signal transduction (reviewed by Frank, 2002).

GH-dependent negative feedback was not observed at somatotrophs in sheep when injected intravenously or intracerebroventricularly with human or bovine GH (Spencer, 1997). These findings, in association with previous studies from Spencer *et al*. (1991 cit. by McMahon *et al*., 2001), seem to suggest that the basal secretion of oGH is not influenced by GH feedback.

GH signalling inhibition could be enhanced by the following factors: prolonged insulin treatment via STAT1 and STAT3 inhibition (Xu *et al*., 2005); SOCS (Adams *et al*., 1998; Cooney *et al*., 2002; Greenhalgh and Alexander, 2004; Greenhalgh *et al*., 2005; Ram and Waxman, 1999), signal regulatory protein-α1 (SIRPα1; Stofega *et al*., 2000), SHP1 and SHP2 (Ram and Waxman, 1997; Rui *et al*., 2000b) via decreasing JAK2 activation; platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA) in a PKC-dependent pathway (Rui *et al*., 2000a); Grb-10 with reduced c-fos and Spi2.1 transactivation (Moutoussamy *et al*., 1998); and via ubiquitin/proteasome pathway required for GHR endocytosis/degradation (Strous and van Kerkhof, 2002; van Kerkhof and Strous, 2001).

Detailed information on the mechanisms involved in GH signalling inhibition has been reviewed by Frank (2001) and Flores-Morales *et al*. (2006).

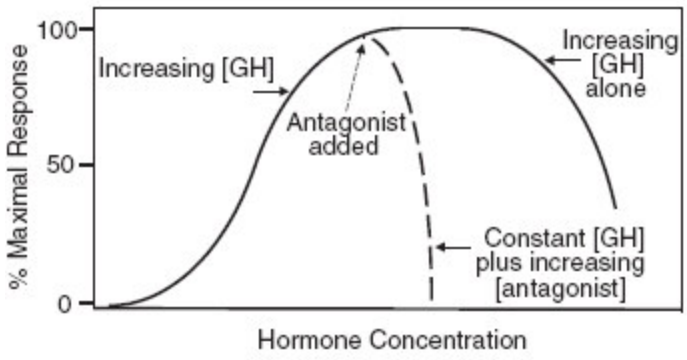

Figure II-9. *GHR-expressing cells' in response to GH or GH plus GH-antagonist treatment. Adapted from Frank and Messina (2002).*

II.6 GH and milk production

Milk production potential depends on the mammary cell differentiation in puberty, pregnancy and early lactation, on the secretory rate of differentiated mammary cells and on cell death (apoptosis) throughout lactation (Pollott, 2000, 2002). Daily expression of the animal's dairy potential depends also on the daily nutrient supply to the mammary gland. This complex

process is under the control of several genes (**Figure II-10**), some of them still unknown. Among them, GH was shown to be essential for mammary development both in the pubertal phase and during pregnancy (Feldman *et al*., 1993; Purup *et al*., 1993; Sejrsen *et al*., 1999). GH also coordinates processes involving alterations in the mammary gland that result in greater rates of milk synthesis and an improved maintenance of mammary cell numbers (Bauman and Vernon, 1993; Etherton and Bauman, 1998; Burton *et al*., 1994).

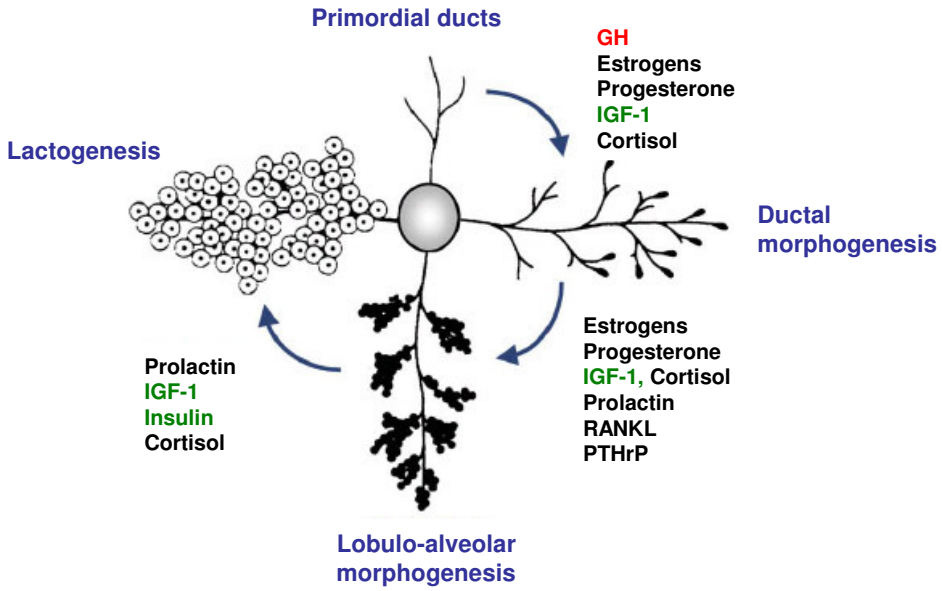

Figure II-10. *Hormonal regulation of the mammary gland development.*
Growth factors involved in each phase of the mammary gland development with emphasis on GH direct influence (in red) or GH indirect influence through IGF-1 stimulation (in green). Adapted from Touraine and Goffin (2005).
GH – growth hormone; IGF-1 – insulin-like growth factor 1; PTHrP – PTH-related peptide; RANKL – nuclear factor-κB receptor activator ligand.

II.6.1 Mammary gland development

Mammary gland development begins during the embryonic life of the females. However at birth the mammary gland is nothing more than a rudimentary organ. Until puberty, the ductal network increases, but only with the onset of puberty does the ductal network develop completely to fill the entire fat pad in the mature female. The lobulo-alveolar morphogenesis takes place during pregnancy, transforming the mammary gland into a functional organ where lactogenesis occurs (**Figure II-10**). After the lactation peak, the last phase of mammary development – involution – occurs, and lobulo-alveolar structures enter in senescence leading to the end of milk production. The knowledge available today about these complex processes and their signalling pathways came from *in vitro* or *in vivo* studies with knockout mice or studies in which orthologous or paralogous GH was injected into ruminants (reviews from Brisken, 2002; Capuco *et al*., 2003; Hennighausen and Robinson, 2001, 2005;

Kelly *et al*., 2002; Neville *et al*., 2002 and Touraine and Goffin, 2005). In this review, emphasis will be given to the effect of GH during mammary gland development and lactation metabolism.

II.6.1.1 From birth to partum

The presence of GH is necessary for the formation of normal mammary gland ducts in the prepuberal phase (Kelly *et al*., 2002). During ductal morphogenesis, GH binds to stroma GHRs stimulating IGF-1 secretion and influencing the epithelial compartment in a paracrine way (Hovey *et al*., 2002 cit. by Neville *et al*., 2002). Until recently, it was considered the major effect of GH in mammary development. However,

- the observed increase in mammary parenchymal cell numbers in primigravid ewes injected with bovine somatotropin (exogenous bGH, bST) (Stelwagen *et al*., 1993);
- the tendency to higher mammary DNA concentration associated with higher levels of plasma GH and IGF-1 and higher milk production in ewes artificially induced to lactate and injected with hGHRH (Kann, 1997; Kann *et al*., 1999); and
- the discovery of GHR in the mammary epithelium as well as in stroma in mice and sheep (Gallego *et al*., 2001; Jammes *et al*., 1991; Ilkbahar *et al*., 1999; Chun *et al*., 2005),

suggest that GH may play other direct roles in mammary development during pregnancy and lactogenesis. Indeed, mammary *GH* gene expression induced by endogenous progesterone levels across the oestrous cycle was observed in normal human and dog mammary glands during the proliferation phase of epithelial cells (Lantinga-van Leeuwen *et al*., 1999; reviewed by van Garderen and Schalken, 2002), and also in sheep induced to lactate after treatment with progesterone and estradiol (Kann, 1997). Moreover, higher levels of progesterone in twin-bearing ewes (Nanalu and Sumaryadi, 1998 cit. by Manalu *et al*., 1999) and in superovulated Javanese thin-tail ewes were correlated with a higher growth of mammary ductal system in the early phase of pregnancy (Manalu *et al*., 1999) and with higher mammary gland synthetic activity (Frimawaty and Manalu, 1999) which consequently increased milk production. Whether *GH* gene is expressed or not in the mammary gland in ovine is still unknown.

GHR and GHBPs were found to increase throughout pregnancy until the onset of lactation in Préalpes du Sud ewes mammary cells and their location change over time (Chun *et al*., 2005), similarly to what was previously reported in mice (Ilkbahar *et al*., 1995). In ovine, GHR-like immunoreactivity was found in the epithelial cells from alveoli at the 90th day of

pregnancy, in plasma membrane and cytosol of the epithelial cells at the 140th day of pregnancy, and in the apical part of the alveolar cells, near the alveolar lumina, at the 3th day of lactation (Chun *et al*., 2005). Furthermore, it was also observed that GH could activate STAT5 and MAPK pathways in ovine mammary acini. These finding suggest that GH could act directly upon mammary growth and lactogenesis through its own receptors, PRLR and/or GHR-PRLR heterodimers (Herman *et al*., 2000).

II.6.1.2 Throughout lactation

During lactation, milk is synthesised within the epithelial cells, secreted to the alveolar lumen and then sent to the mammary gland cistern by a system of ducts. Milk production depends on epithelial cell number and on their activity level. As lactation peak is reached around the third week of lactation in ewes (Cardellino and Benson, 2002; Delgado and Martin, 1992; Ribeiro, 1999; Ruiz *et al*., 2000), some increase in mammary cell number or in mammary cell activity occurs during early lactation (Tucker, 1981), depending on the species. For instance, an increase in secretory cell number was observed before the lactation peak in goats, which accounts for the increased milk production on that lactation period (Knight and Peaker, 1984 cit. by Capuco *et al*., 2003). But in dairy cows, the increased milk production that occurs at the referred lactation phase seems to be the result of the enhanced activity of the cells only (Capuco *et al*., 2001). After the lactation peak, the rapid drop in milk production, and consequently reduced lactation persistency, appears to be related with the decline in mammary epithelial cell number in cows (Capuco *et al*., 2001). So, the lactation persistence depends on the ratio between cell proliferation and cell death (apoptosis; see Capuco *et al*., 2001; 2003).

The administration of ST is one of the factors pointed out to improve lactation persistency (Baldi *et al*., 2002; Bauman *et al*., 1999; Capuco *et al*., 2001; Gallo *et al*., 1997). It appears to act via two mechanisms, a direct and/or indirect stimulus upon cell proliferation, and an indirect inhibition of cell apoptosis mediated by IGF-1 (Forsyth, 1996) and plasminogen/plasmin system (Politis *et al*., 1990). Indeed, it was observed that IGF-1 promotes the ductal system and acini development in mammary cell cultures throughout lactation (Plaut *et al*., 1993; Dallard *et al*., 2005). However, IGF-1 action is controlled by a loop mechanism whereby it could be inhibited (Sejrsen *et al*., 1999; Berry *et al*., 2001) or enhanced (Grill and Cohick, 2000 cit. by Cohick *et al*., 2000) by IGF-binding protein type 3 (IGFBP-3) depending on the nutritional status of the animal (Vestergaard *et al*., 1995; Sejrsen *et al*., 2000) and on the lactation stage (Sejrsen *et al*., 2001) and, together with cAMP, could regulate *IGFBP-3* gene expression (Vestergaard *et al*., 1995; Cohick *et al*., 2000).

Table II-2. *Biological effects of GH in farm animals during lactation.*

Tissue	Physiological process affected [a]
Mammary tissue (lactation)	↑ synthesis of milk with normal composition ↑ synthesis of lactose ↑ uptake of nutrients used for milk synthesis NC GLUT1 mRNA ↑ activity per secretory cell ↑ maintenance of secretory cells, i.e. ↓ involution ↑ blood flow consistent with change in milk synthesis
Adipose tissue	↓ lipogenesis if in positive energy balance ↑ lipolysis if in negative energy balance ↓ glucose and acetate uptake and glucose oxidation ↓ GLUT4 mRNA ↓ insulin stimulation of glucose metabolism and lipid synthesis ↑ catecholamine stimulation of lipolysis ↓ antilipolytic effects of adenosine and prostaglandins
Liver	↑ basal rates of gluconeogenesis ↑ ability to synthesize glucose ↓ ability of insulin to inhibit gluconeogenesis
Kidney [b]	↑ production of 1,25-vitamin D_3
Intestine [b]	↑ absorption of calcium and phosphorus required for milk (lactation) ↑ ability of 1,25-vitamin D_3 to stimulate calcium binding protein ↑ calcium binding protein
Skeletal muscle	↓ glucose uptake ↑ lactate output ↓ glucose oxidation (inferred) ↓ insulin receptor abundance and tyrosine kinase activity [b] ↓ GLUT4 mRNA
Pancreas	NC basal or glucose-stimulated secretion of insulin NC basal or insulin/glucose-stimulated secretion of glucagon
Systemic effects	↓ glucose oxidation ↓ glucose response to insulin tolerance test ↑ NEFA oxidation if in negative energy balance ↓ amino acid oxidation and blood urea nitrogen ↑ circulating IGF-1 and IGFBP-3, and ALS ↓ circulating IGFBP-2 and IGFBP-5 ↑ cardiac output consistent with increases in milk output ↑ enhanced immune response NC energy expenditure for maintenance NC partial efficiency of milk synthesis ↑ voluntary intake to mach nutrient needs for extra milk synthesis ↑ productive efficiency (milk/unit of food intake) ↓ animal waste (faecal and urine output/unit of milk

[a] Changes (↑– increase; ↓ – decrease; NC – no change) that occur in initial period of bST treatment; ALS - acid-labile subunit; GLUT1 – erythrocyte-type glucose transporter; GLUT4 – insulin-responsive glucose transporter; IGF-1 - insulin-like growth factor 1; IGFBP – insulin-like growth factor binding protein; NEFA - non-esterified fatty acids.

[b] Demonstrated in non-lactating animals and consistent with observed.

Adapted from Bauman (1992, 1999), Bauman and Vernon (1993), Bell and Bauman (1997), Chilliard *et al.* (1998a), and Etherton and Bauman (1998).

The increased persistency of lactation after treatment with ST could also be related with the GH-induced inhibition of the IGFBP-5 levels, since IGFBP-5 has been shown to induce mammary cell apoptosis in dairy cows (Accorsi *et al*., 2002) and in rodents (Allan *et al*., 2002; Tonner *et al*., 2002). In recent reviews it was postulated that the effects of IGFBP-5 could be mediated by IGF-independent pathways involving cross-relationships between some elements of the plasminogen/plasmin system and the matrix metallo-proteinases that participate in tissue remodelling during involution [reviewed by Allan *et al*. (2004) and Flint *et al*. (2005)].

The maintenance of mammary cell number and activity has also been shown to be related with external physical factors such as increasing milking frequencies/reducing milking intervals (Boutinaud *et al*., 2003; Bryson *et al*., 1993; Stelwagen *et al*., 1994; Vetharaniam *et al*., 2003).

II.6.2 Mechanisms of GH action during lactation

The somatotrophic axis plays a key role in the coordination of lipid, carbohydrate and protein metabolism in mammals, with GH being direct or indirectly involved in it, contributing to the homeorhetic control of the metabolism by regulating homeostatic signals (Bauman and Currie, 1980). In this section, the effects of GH upon ruminant metabolism during lactation will be discussed. Biological effects of GH during lactation are summarised in **Table II-2**.

II.6.2.1 Lipid metabolism

GH has a major impact on lipid metabolism but its effects depend on the nutritional and physiological status of the animals. In late pregnancy and early lactation, animals are usually in negative energy balance, having high energy needs. During that period, the levels of putative homeorhetic hormones change (**Figure II-11**) in order to adjust body metabolism to overcome those needs.

In early lactation (before peak), when ewes are in negative energy balance, the high levels of endogenous GH stimulate lipolysis. This seems to be related to increased response and sensitivity to catecholamines via increasing numbers of β-adrenergic receptors, adenylate cyclase enhanced activity and increased amounts of G_s-protein α-subunits (Vernon *et al*., 1995), with an increase (McDowell *et al*., 1987; Rose *et al*., 2005) or no change in non-esterified fatty acids (NEFA) levels (Chilliard *et al*., 1998b). Surprisingly, lactation also increases the response to adenosine, an anti-lipolytic factor (Vernon *et al*., 1991b). After peak, when the animal needs for milk production begin to decrease and energy balance becomes zero or positive, administration of ST induces metabolic changes in the adipose tissue resulting in increased

lipolysis. However, that is presumably due to a decrease in the anti-lipolytic activity of adenosine rather than to an increase in β-adrenergic response (Houseknecht and Bauman, 1997; Lanna *et al*., 1995). ST action reflects on adipose tissue response to insulin and in the activity of the lipogenic enzymes.

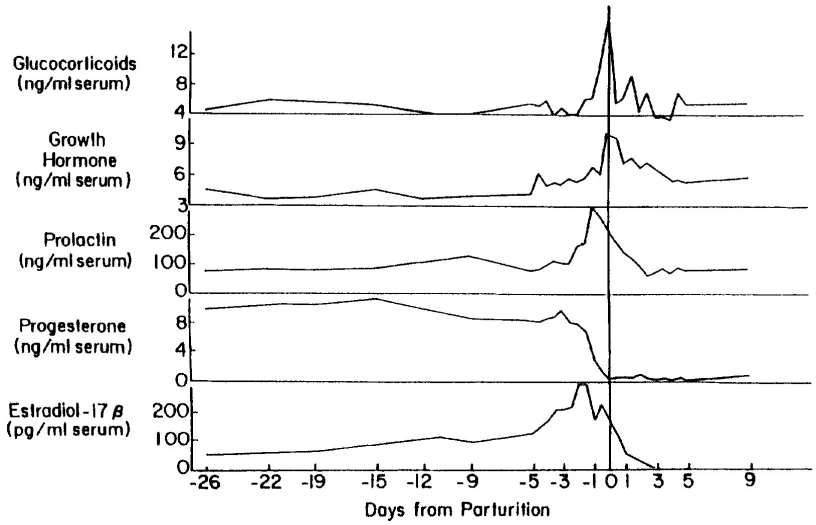

Figure II-11. *Changes in blood serum concentrations of putative homeorhetic hormones in cows. Adapted from Tucker (1985).*

ST treatment is frequently associated with insulin resistance, i.e., a diminished biologic response of tissues to insulin (Etherton and Bauman, 1998). GH regulates SOCS mRNA expression in 3T3-L1 adipocytes via JAK/STAT-signalling pathways inducing insulin resistance (Fasshauer *et al*., 2004). In the presence of insulin resistance, impaired activation of insulin receptor, insulin receptor substrate proteins and phosphatidylinositol 3-kinase has been observed, which in the end may result in the disruption of insulin-induced metabolic targets, such as glucose uptake (Kahn & Flier, 2000 cit. by Fasshauer *et al*., 2004). It has been demonstrated that GH stimulates SOCS-3 mRNA expression in liver and in 3T3-L1 adipocytes (Adams *et al*., 1998) and that SOCS-1, SOCS-3 and SOCS-6 are strong inhibitors of insulin signalling (Emanuelli *et al*., 2000; Mooney *et al*., 2001; Rui *et al*., 2002).

In studies where ST was administrated to dairy cows, it was observed that increased lipolysis was possibly due to a moderate increase in hormone sensitive lipase activity. Simultaneously, ST treatment induced lower adipose tissue lipogenesis mediated by a reduction in acetyl-coenzyme A and fatty acid synthase enzymatic activities (Beswick and Kennelly, 1998; Lanna *et al*., 1995; Vernon *et al*., 1991a), and to a lesser extent a reduction in glucose-6-phosphate dehydrogenease and isocitrate dehydrogenease enzymes activities (Lanna *et al*., 1995).

II.6.2.2 Carbohydrate metabolism

As mentioned in **Table II-2**, GH influences carbohydrate metabolism in several tissues during lactation. Glucose availability at the mammary gland level is a limiting factor in milk synthesis, since it is the key precursor for lactose synthesis at the mammary epithelial cells (Neville *et al*., 1983 cit. by Zhao *et al*., 1996). In ruminants, the main source of glucose is the hepatic gluconeogenesis that uses mainly the propionate originated by rumen microbial fermentations (Bauman and Elliot, 1983). Indeed, hepatic phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression has been shown to be higher in dairy cows receiving ST, presumably reflecting a major ability for gluconeogenesis from gluconeogenic precursors, namely propionate, than from oxaloacetate under ST treatment (Velez and Donkin, 2004), as it was observed that the conversion rate of propionate to glucose increased in cows receiving ST without changes on the conversion rate of propionate to succinate, malate, and oxaloacetate taken together (Knapp *et al*., 1992 cit. by Velez and Donkin, 2004). Authors also postulate that the increased PEPCK expression could be a sign of hepatic insulin insensitivity that has been described in ruminants.

It has not been unanimously demonstrated that higher extracellular glucose concentration could influence glucose mammary uptake in lactating ruminants (Miller *et al*., 1991; Peel *et al*., 1982). Accordingly, a sodium-dependent and a facilitative family of tissue-specific glucose transporters have been proposed to regulate glucose uptake in cells (Gould and Holman, 1993 cit. by Zhao *et al*., 1996a). Facilitative glucose transporters family, which transport glucose according to its concentration gradient, mediate glucose withdrawal from liver, kidney and intestine cells to plasma (reviewed by Hocquette and Abe, 2000). ST and GHRH administration favours the repartitioning of glucose to the mammary gland by decreasing significantly the expression level of the insulin-responsive glucose transporter gene (*GLUT4*) in skeletal muscle, with the same tendency observed at the omental fat tissue (Zhao *et al*., 1996b). However, no changes were observed upon erythrocyte-type glucose transporter (*GLUT1*), liver-type glucose transporter (*GLUT2*) and intestinal-type glucose transporter (*GLUT5*) mRNA expression levels in liver or in kidney (Zhao *et al*., 1996a). GLUT1 mRNA expression seems to be dominant in lactating bovine mammary tissue (Zhao *et al*., 1993 cit. by Nielsen *et al*., 2001). It was thus suggested that glucose uptake in ruminant mammary gland occurs by an insulin-independent mechanism in which glucose transport could probably be done by GLUT1 (Komatsu *et al*., 2005; Nielsen *et al*., 2001). Those results, taken together with others suggest that the ST's effects upon milk production may involve: an increase in mammary glucose uptake due to a higher mammary blood flow rather than changes in glucose

transporters; and an enhanced lactose synthesis through a more efficient intracellular glucose metabolism as proposed in goats by Nielsen *et al*. (2001).

II.6.2.3 Protein metabolism

High levels of endogenous GH observed in late pregnancy/early lactation and ST treatment are known to change amino acids and protein metabolism in lactating mammals (Bell *et al*., 1995). Amino acids are fundamental for milk synthesis at the mammary gland during lactation (Bauman *et al*., 1988), being also precursors for hepatic gluconeogenesis along with propionate, lactate and, to a lesser extent, glycerol (see Bell and Bauman, 1997).

The main changes that high levels of GH induce in protein metabolism consist in an increase in body protein mobilization, namely skeletal muscle amino acids. This has been suggested to be concomitant with a possible decrease in amino acid catabolism and an increase in hepatic glucose and protein synthesis, as well as a more efficient mammary gland milk protein synthesis (Reynolds *et al*., 1994). A decrease in amino acid catabolism was associated with reduced levels of plasma urea nitrogen (Morris *et al*., 1992 cit. by Velez and Donkin, 2004). This was observed in several studies in lactating cows (Sechen *et al*., 1989), goats (Disenhaus *et al*., 1995) and ewes (Sallam *et al*., 2005) treated with ST, but was not observed in others studies (e.g., Velez and Donkin, 2004).

The mechanism by which GH promotes amino acid mobilization in skeletal muscle, and simultaneously increases milk protein synthesis in mammary gland, appears to involve insulin receptor signalling impairment at organs other than mammary gland, and enhanced expression of IGF-1 at the mammary gland.

II.6.3 Recombinant somatotropin and milk production in ewes

Since the 50's, ST effects upon ewes' milk yield have been studied (Dracy and Jordan, 1954; Jordan and Shaffhausen, 1954). Lactating ewes treated with ST yielded significantly more milk (Brozos *et al*., 1998; Chiofalo *et al*., 1999; Fernandez *et al*., 1995, 1997, 2001; Leibovich *et al*., 2001; Min *et al*., 1997; Sallam *et al*., 2005; Sandles *et al*., 1988) (**Figure II-12**). This was also achieved by giving GHRH to ewes in artificially induced lactation (Kann, 1997). Another way to increase milk yield in ewes is through the immunization of pregnant ewes against SRIF (Sun *et al*., 1990; Westbrook *et al*., 1993). Accordingly, milk production in ewes seems to be regulated by genes from the somatotrophic axis as it was also demonstrated in dairy cows (Bauman, 1999; Chilliard *et al*., 1998a; Etherton and Bauman, 1998; Rose *et al*., 2005) and goats (Boutinaud *et al*., 2003; Gallo *et al*., 1997).

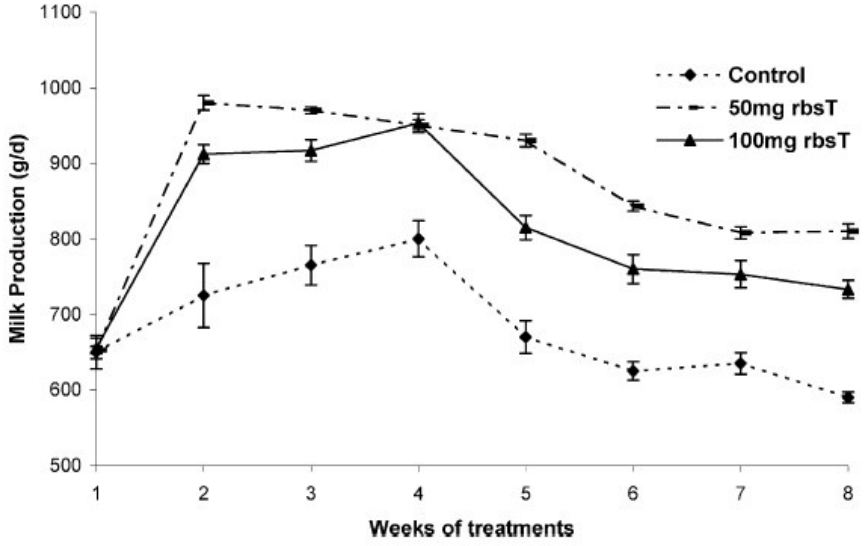

Figure II-12. *Milk production in lactating ewes submitted to recombinant bovine ST (rbsT) treatment. Adapted from Sallam* et al. *(2005).*

Factors such as lactation number and stage, flocks' average milk production, animals' body condition score and weight at the beginning of the treatment influence the ST dose that should be given to the ewes (Fernandez *et al*., 1997) and at which time intervals (Fernandez *et al*., 2001).

In general, the use of recombinant ST for ewes' milk production allows to obtain higher productions with no detrimental effects on milk gross composition and coagulating properties (except in the advanced stage of lactation; Baldi, 1999), which is of great importance for cheese manufacture. Nevertheless, as far as the application of this technology to dairy cows is concerned, 79 % of the UK dairy farmers and 65.4 % of the UK consumers did not consider bST 'ethically acceptable' (Millar *et al*., 1999 cit. by Mepham, 2000). These acceptability problems are expected to arise in respect to dairy sheep industry. The immunization of pregnant ewes against SRIF as proposed by Westbrook *et al*. (1993) has the advantage of obtaining more milk without injecting the animals during the lactation period, and thus without the negative impact on consumer's opinion.

With regard to animals' health, ST treatment did not change hematological parameters (Sallam *et al*., 2005), mastitis incidence or milk somatic cell count (Fernandez *et al*., 1995). However, in dairy cows some increase in milk somatic cells count was attributed to the higher milk production (see Chilliard *et al*., 1998a). It was also reported a higher occurrence of foot and leg disorders in cows subjected to long-term treatment with ST (Zhao *et al*., 1992; Collier *et al*., 2001), which may also occur in ewes since GH axis has been seen to promote bone growth (Braithwaite, 1975). The above mentioned health effects upon females injected with

bST resulted in increasing culling rates in multiparous cows (European Commission, 1999a cit. by Mepham *et al*., 2000), thus increasing milk production costs. This correlates with studies that reported that GH deficiency due to *GH* gene deletion or to combined pituitary hormone deficiencies - as observed in Ames dwarf mice (*PROP1* mutation; Bartke and Brown-Borg, 2004), in Snell dwarf mice (*POU1F1* mutations; Lin *et al*., 1994) - or deficient transduction of GH signal - as in Laron mice (*GHR* knock-out; Coschigano *et al*., 2003), which usually result in lower body weight and decreased insulin and IGF-I levels. As a consequence, the GH deficiency improves lifespan.

II.7 Transgenic animals expressing an additional GH copy

Due to its effects on animal metabolism and its proved efficiency in increasing productivity in animal husbandry, GH is a natural target for genetic manipulation in livestock. Experiments in this field were performed in sheep (Adams *et al*., 2002; Adams and Briegel, 2005). The authors reported that, when expressed, the impact of the transgene depends on age, breed and sex of the transgenic animal. Nevertheless, animals expressing an additional *GH* gene presented a plasma GH level twofold higher that the non-transgenic animals, but GH expression was continuous. Transgenic animals were leaner, grew faster and had similar wool productions (Adams *et al*., 2002). Ewes had greater ovulation and foetal death rates (Adams and Briegel, 2005). In addition, health problems such as higher parasite faecal egg count and foot problems (swollen metatarsal and metacarpal joints) were present in transgenic animals (Adams *et al*., 2002; Adams and Briegel, 2005). Skeletal malformations had been observed before in transgenic sheep (Ward *et al*., 1990), and foot problems observed in dairy cows injected with bST, accounting for the reduced lifespan of those animals (Zhao *et al*., 1992; Collier *et al*., 2001).

II.8 Impact of GH *polymorphisms on productive traits*

In the 90's, with the huge development of the molecular biology techniques, scientists begun to search more actively for polymorphisms at the DNA level that might be responsible for alterations of gene functions leading to changes that could be involved in a disease situation or impair/enhance a production trait. Several techniques were developed and refinements are proposed every day, and applied to domestic animals to look for polymorphisms at candidate

genes that are suspected to influence a particular production trait. Examples of these techniques are: restriction fragment length polymorphism (RFLP; Beckmann and Soller, 1983 cit. by Valinsky *et al*., 1990), SSCP (Orita *et al*., 1989) and single nucleotide polymorphism (SNP; reviewed by Kwok and Chen, 2003); or in genomic regions that might be close to a gene that regulates a production trait (quantitative trait loci; QTL), e.g. microsatellites (Weber and May, 1989), random amplified polymorphic DNA (RAPD; Williams *et al*., 1990) and amplified fragment length polymorphism (AFLP; Vos *et al*., 1995).

GH gene polymorphisms were shown to be a possible selection criterion for milk production traits of high merit animals, mostly in dairy cattle (Lucy *et al*., 1993; Falaki *et al*., 1996b; Lagziel *et al*., 1996). Small ruminant species have been much less studied.

Polymorphisms at the *GH* gene were identified in bovine: two insertion/deletion in the promoter region (ins/del AAG - Rodrigues *et al*., 1998; ins/del TGC – Yao *et al*., 1996); a *Msp*I-RFLP at the intron 3 (Zhang *et al*., 1993; Lagziel *et al*., 2000; Khatami *et al*., 2005); two SNPs detected by SSCP in intron 4 (Lee *et al*., 1994b); an *Alu*I-RFLP at the exon 5 (Lucy *et al.,* 1991; Zhang *et al*., 1993; Chrenek *et al*., 1998b); a *Hae*III-RFLP at the exon 5 (Unanian *et al*., 1994); and *GH* gene haplotypes established by SSCP (Lagziel and Soller, 1999). It was also described a splicing variant of the b*GH* gene which results from nonsplicing of the intron 4 (Hampson and Rottman, 1987). Some of the polymorphisms were identified in several breeds and associations with meat and milk production traits, metabolic parameters and reproduction traits were established (**Table II-3**), sometimes reaching contradictory conclusions.

Table II-3. *Polymorphisms at the GH gene significantly associated with production traits and metabolic parameters in bovines.*

Site	Polymor-phisms	Breed	Effect	References
L127V (exon 5)	*Alu*I-RFLP	Holstein, Brown Swiss, Guernsey, Jersey, and Ayrshire	Estimates of transmitting ability for milk production tended to be: • greater for LL Holstein cows; • greater for VV Jersey cows; • no differences for Holstein sires.	Lucy *et al*., 1993
L127V (exon 5)	*Alu*I-RFLP	Holstein	*Alu*I(+/-), i.e., the presence of V allele: • ↓ 170 kg of EBV-milk and • ↓ 240 kg of AYD-milk	Lee *et al.,* 1996
L127V (exon 5)	*Alu*I-RFLP	Simmental	VV genotypes: • ↓ body weight; • ↓ average daily gain.	Chrenek *et al*., 1998a
L127V (exon 5)	*Alu*I-RFLP	Simmental	LV genotypes: • ↑ carcass gain.	Schlee *et al*., 1994a

Table II-3. *Polymorphisms at the GH gene significantly associated with production traits in bovines and metabolic parameters (cont.).*

Site	Polymor-phisms	Breed	Effect	References
L127V (exon 5)	*Alu*I-RFLP	Black and White	LL genotypes: • ↑ concentration of GH; • ↓ concentration of IGF-1.	Schlee *et al*., 1994b
L127V (exon 5)	*Alu*I-RFLP	Holstein	LV genotype: • More frequent in top ETA bulls.	Sabour *et al*., 1997
L127V (exon 5)	*Alu*I-RFLP	Polish Friesian	No significant effect on overall parameters of GH release. VV genotypes: • ↑ GH baseline in heifers and bulls; • ↑ GH peak amplitude in bulls.	Grochowska *et al*., 1999
L127V (exon 5)	*Alu*I-RFLP	German Black and White, Yaroslavl	LV genotypes: • ↑ milk fat content.	Khatami *et al*., 2005
L127V (exon 5)	*Alu*I-RFLP	Several	VV bulls tendency: • ↑ ejaculate volume; • ↑ day 60 non-return rates.	Lechniak *et al*., 1999
Intron 3 and 3' region	*Msp*I-RFLP and/or ins/del in 3' region	Holstein	Milk fat yield	Lee *et al*., 1994a
Exon5	*Hae*III-RFLP	Holstein	Linked to a QTL for milk protein content	Vukasinovic *et al*., 1999
Gene haplotypes	SSCP	Israel Holstein	Milk protein content	Lagziel *et al*., 1996
GH gene	*Taq*I-RFLP	Double muscled Belgium White Blue	AA genotype: • ↑ weight at 7 and 13 months	Renaville *et al*., 1994; Sneyers *et al*., 1994
GH gene	*Taq*I-RFLP	Holstein Simmental	Milk yield traits	Falaki *et al*., 1994
GH gene	*Taq*I-RFLP	Italian Simmental	• Not significant in milk yield traits • $EBV_{milk\ yield}$ of BB bulls > AA bulls in with more 382±185 kg • $EBV_{protein\ content}$ of BB > (AA =AB)	Falaki *et al*., 1997
GH gene	*Taq*I-RFLP	Italian Holstein-Friesian	(AA = AB) >AE for milk, fat and protein yield	Falaki *et al*., 1996b
Intron3 and GH gene	*Msp*I- and *Taq*I-RFLP	Italian Holstein-Friesian	No effect milk traits	Falaki *et al*., 1996a
Intron 3, and 3' region	MspI-RFLP and ins/del at 3' region	Red Danish, Norwegian Red	*Msp*I(-) allele/Del allele more frequents in high milkfat line	Hoj *et al*., 1993
Intron 3	MspI-RFLP	Israel Holstein	*Msp*I(+/-) vs *Msp*I(+/+) genotype: • ↑ milk protein content and yield • ↓ Somatic cell count	Lagziel *et al*., 1999
Promoter region	Three SNPs	Angus	• Serum IGF-I concentration on day 42 post-weaning; • mean IGF-I concentration.	Ge *et al*., 2003

AYD – Average yield deviation; EBV – estimated breeding value; ETA – estimated transmitting ability for milk traits.

Some polymorphisms were identified at the o*GH* gene: *Taq*I- and *Pvu*II-RFLP (Gootwine *et al*., 1993, 1996; Parsons *et al*., 1992) and *Eco*RI-RFLP (Barracosa, 1996; Gootwine *et al*., 1998), and PCR-SSCP polymorphisms (Bastos *et al.*, 2001; Santos *et al*., 2004) have been reported.

Recent studies conducted on dairy goats have shown associations between PCR-SSCP at the *GH* gene and milk production traits: polymorphisms at exons 4 and 5 were significantly associated to milk yield in "Algarvia" goats (Malveiro *et al.,* 2001). In another study, it was observed that goats with SSCP pattern A/B at the *GH* exon 2 yielded more milk than the other goats from the "Serrana Jarmelista" ecotype; goats with SSCP pattern A/B at the *GH* exon 4 yielded more milk than the other goats from "Serrana Ribatejano" ecotype; in the "Serrana Ribatejano" ecotype, goats with patterns A/B at the *GH* exon 1 and B/B at the *GH* exon 2 had higher milk protein content than the other goats (Marques *et al*., 2003).

III.

MATERIALS and METHODS

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie (1867-1934)

III.1 Serra da Estrela sheep production system

III.1.1 Geographical area

The animals that are the object of the present study came from commercial farms located in the geographical area of milk production for "Serra da Estrela" cheese (PDO) known as "Demarcated Region of Serra da Estrela Cheese" (DRSEG; **Figure III-1**). The DRSEG includes all the municipalities of Carregal do Sal, Celorico da Beira, Fornos de Algodres, Gouveia, Mangualde, Manteigas, Nelas, Oliveira do Hospital, Penalva do Castelo and Seia; and some parishes in the municipality of Aguiar da Beira, Arganil, Covilhã, Guarda, Tábua, Tondela, Trancoso and Viseu (decrees No. 42/85 and D06/94 Reg EC 12/06).

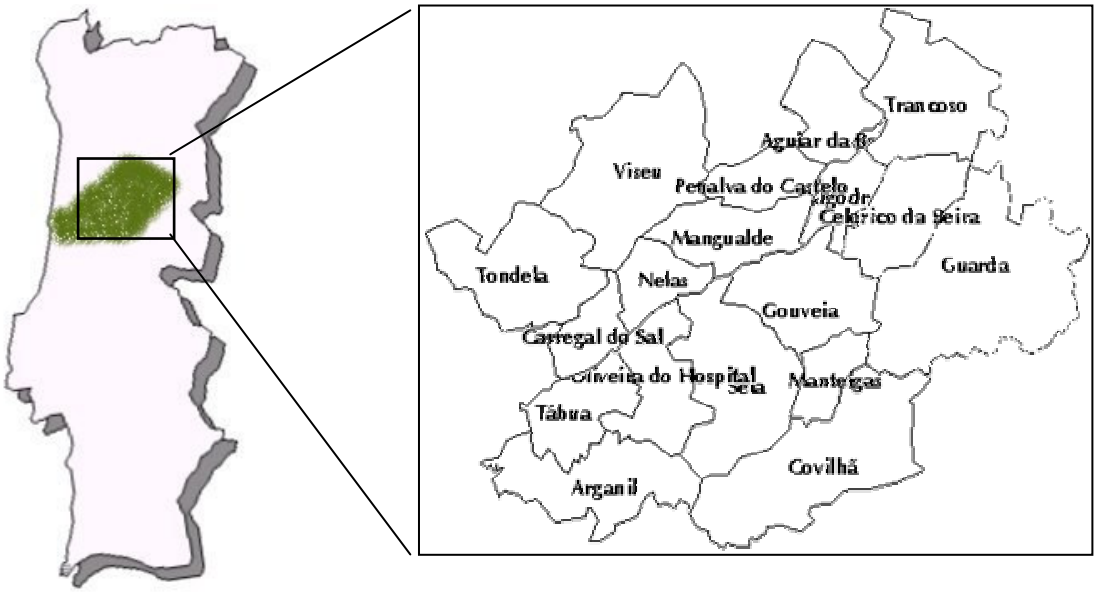

Figure III-1. *Municipalities of the geographical area of the Demarcated Region of the "Serra da Estrela" Cheese (DRSEG).*

III.1.2 Topography and soil types

The DRSEG is located in "Serra da Estrela" mountain region (40º 20' N, 7º 35' W) which is characterized by plateaus of various altitudes and wide valleys (Vieira *et al*., 2006), presenting glacial landscapes.

Geologically, the "Serra da Estrela" is mainly a granite region (Hercynian granites – 300 My; Vieira *et al*., 2006) with large areas of large rocky outcrops. It has also areas of schist-metagreywackes (650-500 My; Vieira *et al*., 2006). The soils are predominantly from types D and E, and present low fertility and profundity (Gulbenkian *et al*., 1993). They are suitable for

forest and pastoral activities. Viera *et al*. (2006) and Natura 2000 report (http://www.eco.science.ru.nl/expploec/Estrela/natura2K.html) reviewed the information concerning topography, geomorphology and vegetation distribution in the "Serra da Estrela" mountain.

III.1.3 Climate

This region has a temperate and humid Mediterranean climate (Le Houerou, 2004), with cold temperatures in winter (often below 0ºC) and hot temperatures in summer (often over 30°C from June to August) with more than 1000 mm of annual rainfall, mainly distributed from October to May and with a high number of rainy days in the western side of the "Serra da Estrela" mountain where a large part of the DRSEG is located.

III.1.4 Sheep production system

In this study a brief characterisation of the "Serra da Estrela" sheep production system was made based on the answers by farmers to the questionnaire presented in **Appendix 1**. An extensive characterization of the "Serra da Estrela" sheep production system had been conducted by Gulbenkian (1993). The questionnaire was answered in 1998 by 36 sheep farmers from municipalities of Oliveira do Hospital, Carregal do Sal and Gouveia. Only farmers who owned brucellosis free flocks (B4) were requested to answer.

Three main aspects were considered:

III.1.4.1 Land utilization

Farms had on average 30 ha (owned and mainly rented land). The foremost occupation of land was the natural (some of them under olive trees) and sown pastures. The forage production practised by the farmers was based upon annual crops. The principal species sown in autumn were rye-grass, rye, oat, and some legumes as clovers and alfalfa and, in spring, sorghum and corn forage ("Milharada"). Pastures were fertilized in all the farms and spring crops were irrigated. Pasture lands were divided in parcels with fences. Generally, the distance between pasture and pen house was covered in less than 30 minutes. Only 15% of the inquired farmers used to graze their sheep in communitary barren lands.

III.1.4.2 Feeding system

One of the most important factors affecting milk production is ewes' nutrition. Throughout the year, feeding regime changes to accommodate the evolution of grass production and the availability of other feeding products in the farm:

- From September to March (autumn/winter): 90% of the flocks graze natural pastures, rye-grass and rye and/or oat, and some legumes. A small percentage of flocks graze shrubs, crops and vineyard remains, and sown pastures.
- From April to August (autumn/winter): ewes graze the remaining of the natural pastures, some communitary pastures in June and above all corn forage ("Milharada") and sorghum.

The ewes receive a feed supplement during milking: a commercial mix in 80% of the flocks with some corn, or rye or oat (250 g to 400 g/ewe). Other flocks receive corn and/or potatoes or a mixture of potatoes and cabbages. Inside the pen overnight, the animals have also rye-grass, oat or natural pasture hay (alfalfa in a very small number of flocks) or oat, rye or corn straw.

Forty percent of the farmers separate the lactating ewes in order to adequate their feeding level to the nutritional needs of the lactation period.

III.1.4.3 Flocks management

Sheep production in the region is mostly a familial activity, with more than 75% of the farmers shepherding their own ewes with the help of a family member. Only less than 25% of the farmers had a shepherd to conduct their ewes to the pastures and to do the milking.

Mating season is in April/May. The lambing season begins in August/September and more than 50% of the multiparous ewes lamb in October. Ewes nurse their lambs 8 to 30 days whereas they are to be sold to be slaughter ("Canastra" lambs). Lambs for flock replacement are weaned at 45-60 days. Ewes are replaced with ewes that are born in the flock, and rams are brought from outside the flock.

III.1.4.4 Milk production and utilization

Milking begins in September/October and ends in June. Ewes are machine (20%) or manually (80%) milked in the morning and evening. The higher milk yield is obtained in October and November. Average milk yields of the inquired flocks are shown in **Appendix 2**. Most farmers (65%) produce milk for sale. "Serra da Estrela" cheese producers need 5.5 to 6 l of milk to manufacture 1 kg of cheese during winter and approximately 5 l of milk during spring. "Serra da Estrela" cheese is a mature cheese prepared by traditional methods from raw milk, and curdled with *Cynara cardunculus.* It has a soft consistency, buttery texture and clean, smooth and slightly acid flavour.

III.2 Animals and milk records

In the present work, 556 "Serra da Estrela" sheep were genotyped. Blood samples (9 ml) were obtained by jugular venipuncture on potassium-ethylenediaminetetracetic acid (EDTA; final concentration of 1.6 mg/ml blood). DNA extraction was performed using a phenol/chloroform free method (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, USA).

The 556 sheep were registered on the "Serra da Estrela" Herdbook. They belong to seven breeders associated in National Association of the Breeders of the "Serra da Estrela" Sheep (ANCOSE) and to ANCOSE Male Testing Center. Pedigree information and official milk records (milk yield and milk quality data) were kindly provided by ANCOSE. Milk yield per lactation was estimated from A4 milking records (ICAR, 2001) using the Fleischmann method and was adjusted for milking length on a reference period of 150 days.

Table III-1. *Flocks' mean milk yield (l/150 days) in the period 1995-2000.*

Flock	n	Variety	Milk yield ± SE
FL1	59	White	92.8 ± 1.7
FL2	71	Black	129.4 ± 1.9
FL3	71	Black	228.2 ± 4.7
FL4	72	Black	90.2 ± 1.4
FL5	116	White	136.9 ± 2.1
FL6	82	White	217.2 ± 3.6
FL7	52	White	218.3 ± 4.1

n – Number of ewes; SE – standard error.

The seven flocks were chosen considering the number of ewes (more than 50), breed variety (white or black) and milk yield level (<100 l/150 days – low; ~130 l/150 days – medium; and >200 l/150 days – high). Number of ewes with milk records within flocks, ewes' variety and flocks' mean milk yield from 1995 to 2000 (1704 valid lactations) are shown in **Table III-1**. Flocks' milk yield; fat and protein content; fat, protein and fat + protein yields in 1998 (the only year with milk composition records – 294 valid lactations) are shown in table **Table III-2**.

Table III-2. *Flocks' mean milk yield (l/150 d), fat content (g/kg), protein content (g/kg), fat yield (kg/150 d, protein yield (kg/150 d) and fat plus protein yield (kg/150 d) in 1998.*

Flock	n	Milk yield ± SE	Fat content ± SE	Protein content ± SE	Fat yield ± SE	Protein yield ± SE	Fat + Protein Yield ± SE
FL1	46	93.4 ± 3.9	73.2 ± 1.3	67.5 ± 1.0	6.9 ± 0.3	6.3 ± 0.3	13.2 ± 0.6
FL2	57	152.3 ± 3.6	81.9 ± 1.2	67.2 ± 0.9	12.6 ± 0.4	10.2 ± 0.3	22.8 ± 0.7
FL3	43	227.0 ± 9.1	78.7 ± 1.6	68.9 ± 1.0	17.9 ± 0.8	15.5 ± 0.6	33.5 ± 1.4
FL4	54	98.9 ± 2.8	76.6 ± 1.4	66.7 ± 0.8	7.6 ± 0.3	6.6 ± 0.2	14.2 ± 0.5
FL6	54	238.0 ± 8.0	82.8 ± 2.0	63.2 ± 0.8	19.5 ± 0.7	15.0 ± 0.5	34.5 ± 1.1
FL7	40	239.3 ± 8.7	83.7 ± 1.8	58.6 ± 1.0	20.1 ± 0.9	14.1 ± 0.6	34.2 ± 1.4

n – Number of ewes; SE – standard error.

*III.3 o*GH *gene copy number genotypes*

To determine the copy number genotype of the o*GH* gene, a total of 89 "Serra da Estrela" sheep (56 ewes and 33 rams) from the white (61%) and the black (39%) varieties were analysed by Southern blotting.

A 2055 bp DNA probe containing the o*GH* gene was amplified by PCR with the primers GHT-Fwd ($^{5'}$ CCA GAG AAG GAA CGG GAA CAG GAT GAG $^{3'}$) and GHT-Rev ($^{5'}$ ATA GAG CCC ACA GCA CCC CTG CTA TTG $^{3'}$) designed according to the published o*GH* gene sequence (GenBank accession number: X12546, Orian *et al.,* 1988). The PCR reaction was performed in a final volume of 50 µl according to the following conditions: 500 ng of genomic DNA; 6 pmoles of each primer; 2.0 unit of *TaKaRa LA Taq*™ (TAKARA SHUZO CO., Ltd, Japan); 1x of 2x GC Buffer I with 2.5 mM $MgCl_2$ and 400 µM of each dNTP. Amplification cycles included an initial denaturation at 94ºC for 1 min followed by 30 cycles of denaturation at 98ºC for 20 s, annealing from 62ºC for 12 min, and a final extension at 72ºC for 10 min. The 2055 bp DNA probe was digoxigenin (DIG)-labelled with the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Indianapolis, USA) according to the instruction manual.

Fifteen µg of genomic DNA were digested overnight, separately, with *Eco*RI, *Bam*HI and *Hind*III restriction endonucleases (Invitrogen Life Technologies; Carlsbad, CA, USA). The digested fragments were separated on a 0.8% agarose gel (2 V/cm) with 0.5x TBE buffer (0.045 M borate, 0.001 M EDTA) for 13 h, denatured in 0.5 M NaOH for 30 min, transferred by capillarity to a positively charged nylon membrane (Hybond™-N^+, Amersham Pharmacia

Biotech, Ireland), and UV-cross linked to the membrane. The blots were hybridized with the 2055 bp DIG-labelled probe at 45ºC in DIG Easy Hyb solution (Roche Diagnostics GmbH, Indianapolis, USA) for 16 h. The probe-target hybrids were immunodetected on the blots with an alkaline phosphatase-conjugated anti-DIG antibody from sheep (Anti-Digoxigenin-AP, Fab fragments; Roche Diagnostics GmbH, Indianapolis, USA) and visualized with the chemiluminescent alkaline phosphatase substrate CSPD (Roche Diagnostics GmbH, Indianapolis, USA). Then the blots were exposed to X-ray film (Kodak BioMax MS1, Eastman Kodak Company, Rochester, NY, USA) for 45 min according to the standard DIG chemiluminescent detection procedure.

The Hardy-Weinberg equilibrium (HWE) for the o*GH* copy number genotypes was tested by χ^2 analysis (Statistica software, StatSoft, Inc., Tulsa, OK, USA).

*III.4 o*GH *gene analysis by PCR-SSCP*

Five hundred and twenty three "Serra da Estrela" ewes from white (64%) and black (36%) varieties were analysed by SSCP to determine o*GH* polymorphisms.

Seven DNA fragments (I to VII) of the o*GH* gene comprising the five exons (including intron-exon junctions), and the 5'-UTR and 3'-UTR regions, were amplified by PCR with copy-unspecific primer pairs (Invitrogen Life Technologies, Barcelona, Spain) shown in **Table III-3**. Sizes of amplified fragments ranged from 112 to 289 bp. PCR reactions of the fragments II to VI were performed in a final volume of 25 µl using Ready-To-Go PCR Beads (Amersham Biosciences, Buckinghamshire, England) according to the following conditions: 25 to 50 ng of genomic DNA; 0.16 to 0.64 µM of each primer; 1.5 units of *Taq* DNA polymerase; 10 mM Tris-HCl (pH 9); 50 mM KCl; 1.5 or 2.5 mM $MgCl_2$; 200 µM of each dNTP and stabilisers including BSA. PCR reactions of the fragments I and VII were performed in a final volume of 25 µl according to the following conditions: 50 ng of genomic DNA; 12 pmoles of each primer; 1.0 unit of *Taq* DNA polymerase (Roche Diagnostics GmbH, Indianopolis, USA); 10 mM Tris-HCl (pH 9); 50 mM KCl; 3.5 mM or 4.5 mM $MgCl_2$; and 200 µM of each dNTP. Amplification cycles included an initial denaturation at 95ºC for 5 min followed by 30 cycles of denaturation at 95ºC for 30 s, annealing from 57ºC to 68ºC for 30 s (**Table III-3**), extension at 72ºC for 30 s and a final extension at 72ºC for 5 min. Amplification products were analysed by electrophoresis on ethidium bromide stained 2% agarose gels (5 V/cm), with 1x TBE buffer (0.09 M borate, 0.002 M EDTA).

Table III-3. *Length and localisation of PCR-SSCP fragments of the oGH gene and primers used for the PCR analysis*[†]

Fragment				Primer		Annealing temperature (°C)
Name	**Type**	**Length**	**Localisation (bp)**	**Name**	**Sequence**	
I	5'-UTR, E1	125	205 to 329	GH5'-Fwd: GH5'-Rev:	5' GGG AAA GGG AGA GAG AAG AAG CCA G 3' 5' CAG CCA TCA TAG CTG GTG AGC TGT C 3'	68
II	5'-UTR, E1, I1	112	248 to 359	GH1-Fwd: GH1-Rev:	5' CAG AGA CCA ATT CCA GGA TC 3' 5' TAA TGG AGG GGA TTT TCG TG 3'	57
III	I1, E2, I2	198	569 to 766	GH2-Fwd: GH2-Rev:	5' CTC TCC CTA GGG CCC CGG AC 3' 5' TCT AGG ACA CAT CTC TGG GG 3'	65
IV	I2, E3, I3	154	967 to 1110	GH3-Fwd: GH3-Rev:	5' CTC CCC CCA GGA GCG CAC CT 3' 5' GCT CCT CGG TCC TAG GTG GC 3'	60
V	I3, E4, I4	200	1303 to 1502	GH4-Fwd: GH4-Rev:	5' CTG CCA GCA GGA CTT GGA GC 3' 5' GGA AGG GAC CCA ACA ATG CCA 3'	60
VI	I4, E5, 3'-UTR	289	1740 to 2028	GH5-Fwd: GH5-Rev:	5' CCC TTG GCA GGA GCT GGA AG 3' 5' AAA GGA CAG TGG GCA CTG GA 3'	67
VII	E5, 3'-UTR	150	1943 to 2092	GH3'-Fwd: GH3'-Rev:	5' CCT TCT AGT TGC CAG CCA TCT GTT G 3' 5' CCA CCC CCT AGA ATA GAA TGA CAC CTA C 3'	64.5

[†] According to the published o*GH* gene sequence GenBank accession number X12546 (Orian *et al.,* 1988).

For SSCP analysis, 4 µl or 5 µl of each amplification product were added to 12 µl or 15 µl of stop solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heat-denatured at 95ºC for 5 min, immediately chilled on ice, and loaded onto native 8-12% polyacrylamide gels, with 2.5% crosslinking and 0.5 or 1x TBE buffer. Gels were run at constant power (25 or 40 W) and temperature (8 to 20ºC), for an optimized time (4 to 9 h) in a DCode™ Universal Mutation Detection System (BIO-RAD, Hercules, USA), coupled to a refrigeration system. After the run, gels were silver stained (PlusOne™ DNA Silver Staining Kit, Amersham Biosciences, Uppsala, Sweden). SSCP patterns were identified and assigned a capital letter.

After cloning and sequencing the o*GH* gene copies and the inter-copy region (see **section III.5**), PCR-SSCP analyses were further carried out on each o*GH* gene copy separately using the previously described conditions. PCR amplicons of the o*GH* copies as well as cloned o*GH* copies were used as DNA templates. Genotypes were assigned to each amplified fragment (I through VII) produced from each gene copy.

III.5 Cloning and sequencing of the oGH gene copies and of the inter-copy region

In order to specifically assign the SSCP bands to the *GH2-N* (or *GH1*) or the *GH2-Z* gene copies, cloning and sequencing of the inter-copy region was performed. A DNA fragment 4527 pb long (ranging from the exon 5 of the *GH2-N* copy to the exon 3 of the *GH2-Z* copy; **Figure III-2**) of a *Gh2*/*Gh2* animal was amplified with primers GH5-Fwd and GH3-Rev (**Table III-3**). The PCR reaction was performed in a final volume of 50 µl according to the following conditions: 250 ng of genomic DNA; 3 pmoles of each primer; 1.0 unit of *TaKaRa LA Taq*™ (TAKARA SHUZO CO., Ltd, Japan); 1x of 2x GC Buffer I with 2.5 mM $MgCl_2$ and 400 µM of each dNTP. Amplification cycles included an initial denaturation at 94ºC for 1 min followed by 30 cycles of denaturation at 98ºC for 20 s, annealing from 60ºC for 12 min, and a final extension at 72ºC for 10 min. The PCR products were column purified, cloned into the pCR®-XL-TOPO® vector according to the instructions of the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies; Carlsbad, CA, USA) and transformed into competent *E. coli* One Shot® TOP10 cells. Recombinant plasmids DNA were purified using the QIAGEN® Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany) following manufacturer recommendations. The 4527 pb long DNA fragment was sequenced from both ends (GenBank

accession number: DQ238053; **Appendix 3).** Sequencing reactions were performed according to the protocol from the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Warrington, England) and repeated for confirmation. The DNA was purified using ethanol precipitation and analysed using an Automatic Sequencer 3730xI.

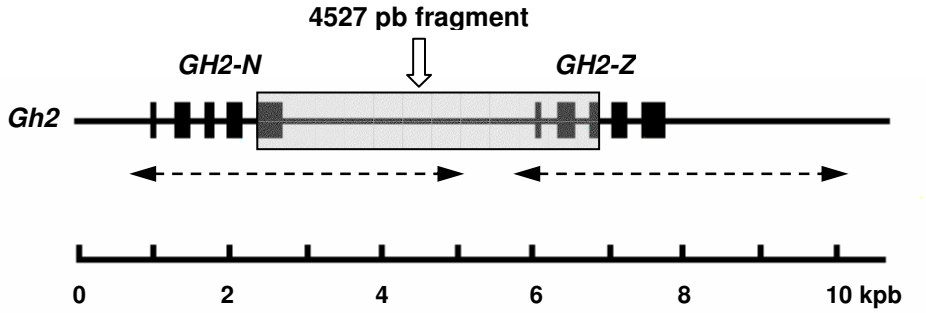

Figure III-2. Gh2 *allele in sheep (following Valinsky* et al.*, 1990) containing the* GH2-N *and* GH2-Z *copies in tandem.*
The black boxes represent the exons. The dashed arrows indicate the supposed duplicated region. The blue box indicates the amplified 4527 pb fragment containing the inter-copies region. Adapted from Wallis *et al.* (1998).

Alignment of the sequences of *GH2-N* and *GH2-Z* gene copies with the inter-copy region sequence using Vector NTI® Suite software (InforMax®, Bethesda, MD, USA) revealed nucleotide variations (see **section IV.2.3**) that allowed the design of primers specific for the *GH2-Z* copy and showed that the GHT-Fwd primer was specific for the *GH1* and *GH2-N* copies. The o*GH* copies of 20 animals, representative of all SSCP patterns, were subsequently sequenced. *GH1* and *GH2-N* copies were PCR amplified using the GHT-Fwd and GHT-Rev primers. The *GH2-Z* copy was amplified using primers GHZ-Fwd ($^{5'}$ GAG GAG TAA ATG AAA TGA GGT C $^{3'}$) and GHZ-Rev ($^{5'}$ CCT CTG TGC TAT GTC CTT CAC AAG C $^{3'}$) designed according to GenBank accession numbers: DQ238053 (our results; **Appendix 3**) and M37310 (Byrne *et al.,* 1987), respectively. The PCR of the *GH1* and *GH2-N* copies was performed as described previously (see **section III.3**). The PCR of the *GH2-Z* copy was performed as described above for the inter-copy region using annealing temperature of 50°C. The PCR products of the o*GH* gene copies were cloned and sequenced as described above and/or directly sequenced after purification using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

All sequences were submitted to the GenBank data base:

- *GH1* copy: GenBank accession numbers DQ450146-DQ450147;
- *GH2-N* copy: GenBank accession numbers DQ461644-DQ461681;

- *GH2-Z* copy: GenBank accession numbers DQ461615-DQ461643.

After alignment of the sequencing data (Vector NTI® Suite software, InforMax®, Bethesda, MD, USA), *GH2-N*, *GH2-Z* and associated *GH2-N* and *GH2-Z* genotypes were established. Haplotypes and their frequencies were inferred using the Family-Based Association Tests software (FBAT; Horvath *et al.,* 2004).

The HWE of the SSCP alleles of *GH2-N* and *GH2-Z* copies was tested with the population genetic software package GENEPOP v3.4 (http://wbiomed.curtin.edu.au/genepop/; Garnier-Gere and Dillmann, 1992; Raymond and Rousset, 1995a, b). Estimation of exact *P-values* was performed by the Markov chain method. Markov chain parameters for all tests were: dememorization – 10000; batches – 100; and iterations per batch – 5000.

III.6 Statistical analysis

III.6.1 oGH copy number genotypes

Two different statistical analyses were performed considering two data sets:

- Data set 1 – milk yield records of the genotyped ewes;
- Data set 2 – milk yield records of the genotyped animals (ewes and rams) progeny.

III.6.1.1 Data set 1 – Milk yield in the genotyped ewes

Data set 1 was statistically analysed to test possible associations between milk yield adjusted to 150 lactation days and copy number genotypes. Data was analysed by restricted maximum likelihood (REML) through univariate analyses with repeated measures using the BLUP - Animal Model and multiple-trait derivative free restricted maximum likelihood analysis (MTDFREML; Boldman *et al.,* 1993). The following model was used:

$$y = X\beta + Z_a a + Z_p p + e$$

where, **y** is the vector of milk records; **β** is the vector of fixed effects which included the effect of year-flock (year 1992 flock 1, year 1997 flock 1, …, year 2001 flock 7), of month of lambing (August, September, …, December), type of lambing (simple or multiple), variety (white or black), the linear and quadratic effect of the ewes' lambing age, and the effect associated with the genotypes studied; **a** is the vector of random additive genetic effects; **p** is the vector of random permanent environmental effects; **e** is the vector of random residual

effects. **X**, $\mathbf{Z_a}$ and $\mathbf{Z_p}$ are the incidence matrixes which relate the fixed (**X**) and random ($\mathbf{Z_a}$ and $\mathbf{Z_p}$) effects with the vector of milk records, **y**. In the relationship matrix **A** 750 animals were considered.

To solve the mixed model equations (MME; **1**) it was assumed that α=2.4, which corresponds to milk production heritability of 0.25 and that γ=4, which corresponds to a repeatability of 0.40.

$$(1) \quad \begin{bmatrix} X'X & X'Z & X'Z \\ Z'X & Z'Z + A^{-1}\alpha & Z'Z \\ Z'X & Z'Z & Z'Z + I\gamma \end{bmatrix} \begin{bmatrix} b \\ a \\ p \end{bmatrix} = \begin{bmatrix} X'y \\ Z'y \\ Z'y \end{bmatrix}$$

In the mixed model equations, **A** is the relationship matrix between all animals (genotyped ewes with milk records and their pedigrees: 750 animals); **I** is the identity matrix; **b** is the solution for fixed effects; **a** is the solution for genetic effects and **p** is the solution for permanent environmental effects, where:

$$\alpha = \frac{\sigma_e^2}{\sigma_a^2} \qquad \text{and} \qquad \gamma = \frac{\sigma_e^2}{\sigma_{pe}^2}$$

Solutions for the effects of the analysed genotypes upon milk yield, contrasts between the analysed genotypes and the corresponding significance test were obtained through option 4 of the subroot MTDFRUN (solutions for MME then sampling variances) from the MTDFREML program (Boldman *et al.,* 1993), using estimates of genetic additive (σ_a^2), permanent environmental (σ_{pe}^2) and residual (σ_e^2) variances of the "Serra da Estrela" ovine population (Department of "Genética e Melhoramento Animal" of the EZN, personnal communication).

Only genotypes *Gh1/Gh2* and *Gh2/Gh2* were considered, as genotype *Gh1*/*Gh1* was only found in one ewe.

III.6.1.2 Data set 2 – Milk yield in the genotyped animals' progeny

Data set 2 was statistically analysed to test possible associations between milk yield adjusted to 150 lactation days and the probability of a ewe to receive allele *Gh2* from its genotyped progenitor (dam or sire). The probability values were 0, 0.5 or 1 depending on whether the progenitor genotype was *Gh1*/*Gh1*, *Gh1*/*Gh2* or *Gh2*/*Gh2*. Data was analysed following two models.

Model 1 was similar to the model described in **section III.6.1.1**, considering the probability of a ewe to receive allele *Gh2* from its genotyped progenitor (dam or sire) as a fixed effect. The fixed effect of the genotyped progenitor was added to the model. . Thus the vector of fixed effects (**β**) included the effect of year-flock (year 1995 flock 1, year 1997 flock 1, …, year 2005 flock 30), of month of lambing (August, September, …, February), type of lambing (simple or multiple), variety (white or black), the linear and quadratic effect of the ewes' lambing age, and the effect of the probability of a ewe to receive allele *Gh2* from its genotyped progenitor (dam or sire). In the relationship matrix **A** 1113 animals were considered.

Model 2 was similar to model 1, but the probability of a ewe to receive allele *Gh2* from its genotyped progenitor (dam or sire) was analysed as a covariate. A regression coefficient (b_1) was obtained for the *Gh2* allele, which corresponds to the milk yield deviation observed for each additional *Gh2* allele received from the progenitor. Regression coefficient was considered significant ($P<0.05$) when b_1 absolute value was higher than twice its respective standard error (SE) (if $|b_1| - 2\times SE > 0$, then $P<0.05$; Boldman *et al*., 1995).

III.6.2 Polymorphism at the o*GH* copies

Data was statistically analysed to test possible associations between milk, fat and protein yields adjusted to 150 lactation days, fat and protein contents and oGH2-N and GH2-Z copies genotypes and phenotypes taken separately or associated. Data was analysed as described in **section III.6.1.1**. The vector of fixed effects (**β**) included the effect of year-flock (year 1996 flock 1, year 1997 flock 1, …, year 2000 flock 7), of month of lambing (August, September, …, December), type of lambing (simple or multiple), variety (white or black), the linear and quadratic effect of the ewes' lambing age, and the effect associated with the genotypes and phenotypes studied. In the relationship matrix **A** 750 animals were considered.

Milk fat and protein contents and milk fat and protein yield were only available from 1998 and in six flocks. Thus, the effect of year-flock was replaced by the effect of the flock only. To solve the mixed model equations (1, see section **III.6.1.1**) a heritability of 0.40 was considered for milk fat and protein contents and of 0.25 for milk fat and protein yields.

III.7 Bioinformatics

The sequences obtained were analysed using specific bioinformatics tools.

III.7.1 Gene finding

Gene finding were performed both by homology and *ab initio* methods. The homology method used was BLASTN (Altschul *et al*., 1997; http://www.ncbi.nlm.nih.gov/BLASTN/), a standard pairwise comparison method which indicates the rough position of exons. The *ab initio* methods used were:

- NetGene2 v2.4 (Brunak *et al*., 1991; Hebsgaard *et al*., 1996; http://www.cbs.dtu.dk/services/NetGene2/) uses artificial neural networks to predict splice sites location.
- GENSCAN 1.0 (Burge and Karlin, 1997; http://genes.mit.edu/GENSCAN.html). It uses generalized hidden Markov models (GHMM) to predict complete gene structures.
- Eukaryotic GeneMark.hmm v2.2a (Lomsadze *et al*., 2005; http://exon.gatech.edu/GeneMark/eukhmm.cgi) with *Homo sapiens* matrix. It uses explicit state duration hidden Markov models (HMMs). The optimal gene candidates, selected by HMM and dynamic programming are further processed by a ribosomal binding site recognition algorithm.

The combined results of the referred methods led to a consensus in exon prediction and allowed sequences annotation and subsequently protein inference.

III.7.2 Transcription factors binding sites

Sequencing results showed that the 5' region of the o*GH* gene is identical in the *GH1* and *GH2-N* copies and different from the *GH2-Z* copy. Thus, a characterization of the regulatory factors involved in the o*GH* gene copies transcription control (~1 kb upstream of the exon 1) was performed. Putative transcription factors binding sites were identified using the PATCH™ public 1.0 pattern search for transcription factor binding sites (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) using TRANSFAC® 6.0 public sites (*BIOBASE* Biological Databases/ Biologische Datenbanken GmbH, Wolfenbüttel, Germany; Matys *et al*., 2003). The TRANSPLORER® site prediction tool uses position weight matrix (Chen *et al*., 1995) and matrices derived from the TRANSFAC® database (http://www.biobase.de/pages/ products/databases.html).

III.7.3 Protein analysis

Conceptual translation of each haplotype was done with Vector NTI® Suite software (InforMax®, Bethesda, MD, USA). Protein motif search was run under PROSITE (http://www.expasy.org/prosite/).

IV.

RESULTS

"Perfect as the wing of a bird may be,
it will never enable the bird to fly if
unsupported by the air.
Facts are the air of science."

Ivan Pavlov (1849 - 1936)

*IV.1 o*GH *copy number genotypes*

IV.1.1 Probe preparation

A 2055 bp DNA probe containing the o*GH* gene was amplified and digoxigenin (DIG)-labelled as described in **section III.3.** The evaluation of probe labelling efficiency was performed through the analysis of PCR-labelled probe by agarose gel electrophoresis (**Figure IV-1**), as labelled product has a significant greater molecular weight than the unlabeled product, due to DIG incorporation into the product.

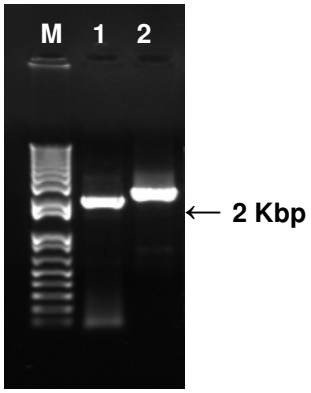

Figure IV-1. *Evaluation of PCR DIG labelling efficiency of the 2055 bp probe in agarose gel electrophoresis.*
The unlabeled PCR product (1) and the corresponding DIG-labelled PCR product (2) were separated by electrophoresis in a 0.8% agarose gel.
M: molecular weight marker

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IV.1.2 o*GH* copy number genotyping

Sheep can be homozygous for one copy (*GH1*, *Gh1* allele), homozygous for the two copies (*GH2-N* and *GH2-Z*, *Gh2* allele) or heterozygous for the copy number (*Gh1* and *Gh2* alleles) (Wallis *et al.,* 1998). Southern blotting analysis of the genomic DNA of the 89 "Serra da Estrela" sheep digested with *Eco*RI showed the three o*GH* genotypes *Gh1*/*Gh1*, *Gh1*/*Gh2* and *Gh2*/*Gh2* (**Figure IV-2**) previously described (Gootwine *et al.,* 1998). The *Gh1*/*Gh1* genotypes were confirmed by *Bam*HI- and *Hind*III-RFLP analysis.

The observed genotypic and allelic frequencies are shown in **Figure IV-3**. The studied population was found in Hard-Weinberg equilibrium (χ^2=0.123; df=2; P>0.10).

Considering only the 56 ewes genotyped for the o*GH* copy number, with available milk yield records, the observed genotypic frequencies were 1.14%, 15.91% and 82.95% for *Gh1*/*Gh1*, *Gh1*/*Gh2* and *Gh2*/*Gh2* genotypes, respectively. Allele frequencies were 9.09% for the *Gh1* allele and 90.91% for the *Gh2* allele. These animals were also found in Hardy-Weinberg equilibrium (χ^2=0.508; df=2; P>0.10).

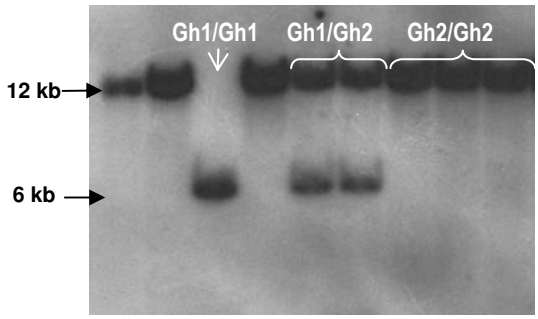

Figure IV-2. Eco*RI-restriction fragment length polymorphism at the growth hormone locus in "Serra da Estrela" sheep.*
Ovine genomic DNA was digested with *Eco*RI, separated on a 0.8% agarose gel, and blotted onto positively charged nylon membranes. The blots were hybridized with 50 ng/mL of a DIG-labeled oGH gene DNA probe. Chemiluminescent detection was according to the standard DIG chemiluminescent detection procedure using CSPD at 0.25 mM final concentration and exposure of the blot to X-ray film for 30 min.

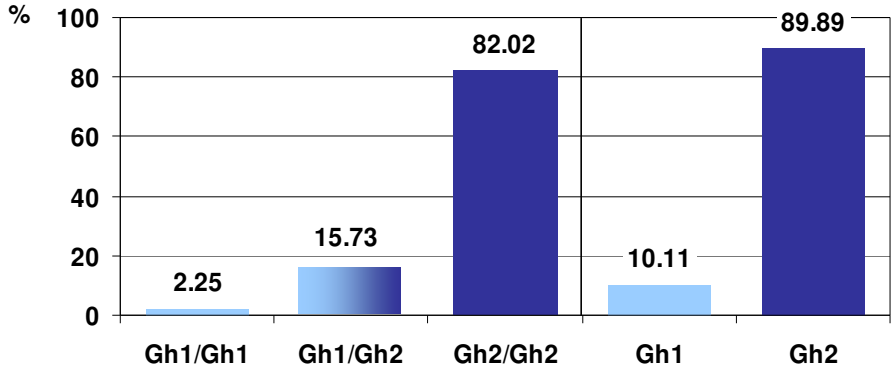

Figure IV-3. *o*GH *copy number genotypic and allelic frequencies in the "Serra da Estrela" sheep.*

IV.1.3 Statistical analysis

IV.1.3.1 Milk yield in the genotyped ewes

The impact of the o*GH* copy number genotype on milk yield adjusted to 150 lactation days was statistically analysed as described **section III.6.1.1**. The number of records used in univariate analyses was 259 lactations from the 56 o*GH* copy number genotyped ewes. The mean milk yield adjusted to 150 lactation days (± SD) was 180.8 ± 90.6 l.

Ewes with *Gh2/Gh2* genotype produced 4.9 ± 8.6 l/150 d more than ewes with *Gh1/Gh2* genotype (P>0.05). Only one ewe of the genotyped population presented the *Gh1/Gh1* genotype, so this genotype was not considered in the analysis.

IV.1.3.2 Milk yield in the genotyped ewes animals' progeny

The impact of the probability of a ewe to receive the *Gh2* allele from either progenitor on milk yield adjusted to 150 lactation days was statistically analysed as described in

section III.6.1.2. The mean milk yield adjusted to 150 lactation days (± SD) was 141.8 ± 60.3 l and the mean daily milk yield (± SD) was 0.94 ± 0.40 l.

The probability of a ewe to receive allele *Gh2* was tested as a covariate and as a fixed effect (**Figure IV-4**). The first analysis (model 1) showed that the ewes which received allele *Gh2* yielded on average 26.4 ± 7.2 l/150 d more than ewes which received allele *Gh1* (P<0.01), and 3.0 ± 8.3 l/150 d more than ewes which could have received either *Gh1* or *Gh2* alleles (P>0.05). The second analysis (model 2) showed that ewes which received allele *Gh2* yielded on average 21.9 ± 7.5 l/150 d more than ewes that received allele *Gh1* (P<0.05). These results suggest that the *Gh2* allele has a strong positive effect upon milk production in "Serra da Estrela" ewes.

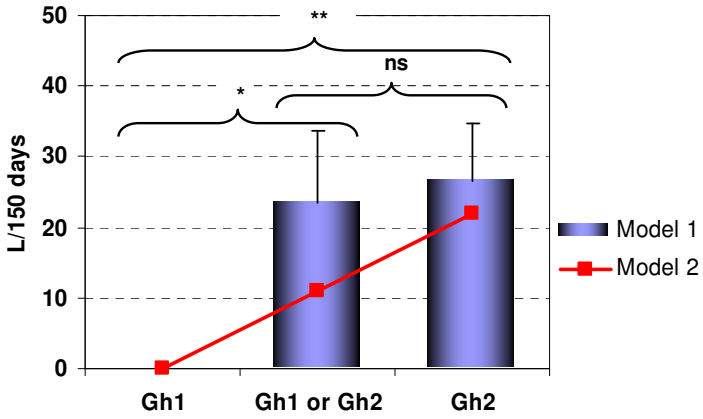

Figure IV-4. *Effect of the probability of a ewe to receive alleles Gh1, Gh2 or either from their progenitor on milk yield adjusted to 150 lactation days (± SE).*
Model 1 – probability of receiving one of the alleles analysed as a fixed effect; **Model 2** - probability of receiving one of the alleles analysed as a covariate; ns - P>0.05; * - P<0.05; ** - P<0.01.

*IV.2 o*GH *gene SSCPs' detection, charaterization and influence on milk traits*

IV.2.1 PCR-SSCP analysis

Seven PCR fragments (I to VII) containing the five o*GH* exons, the 5'-UTR and the 3'-UTR analysed by PCR-SSCP were found to be highly polymorphic (**Figure IV-5**). Except for the 5'-UTR, the primers used in the amplification were not copy-specific due to the high homology of sequences in the 3'-UTR, and in exons and adjacent intronic regions. PCR-SSCP revealed four SSCP patterns in the fragment I, two in fragment II, 17 in fragment III, eight in fragment IV, two in fragment V, five in fragment VI, and seven in fragment VII. SSCP pattern frequencies (total and by flock) are shown in **Table IV-1** and **Table IV-2**. The most frequent

SSCP pattern was denoted as A for all the fragments. Its frequency was found to be 91.88% in fragment I, 93.12% in fragment II, 22.41% in fragment III, 57.36% in fragment IV, 84.70% in fragment V, 48.18% in fragment VI and 36.82% in fragment VII. However, considering each flock separately, the SSCP pattern A was not found to be the most frequent one for some fragments.

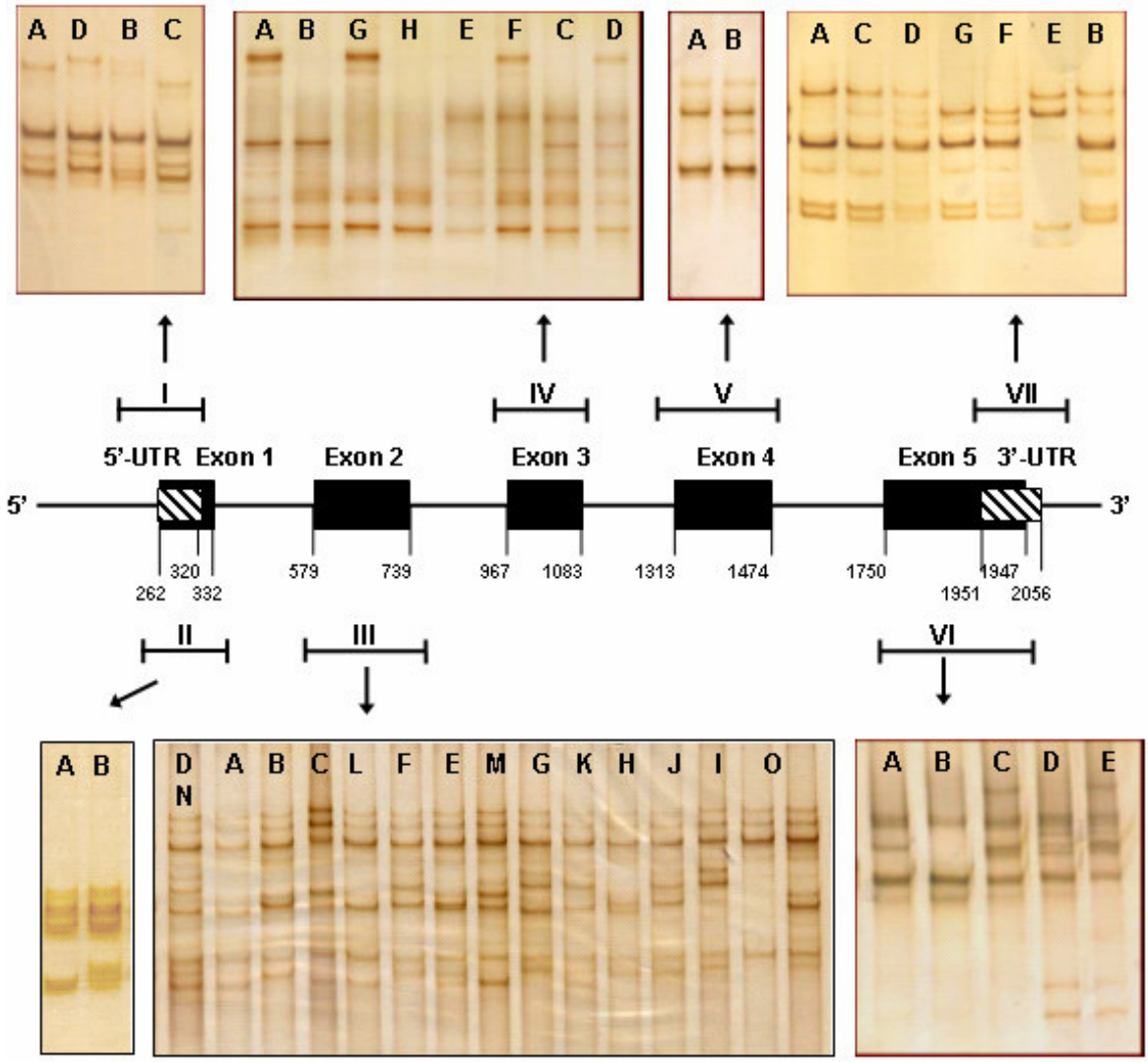

Figure IV-5. *PCR-SSCP patterns for fragments I (5'-UTR, exon 1), II (5'-UTR, exon 1, intron 1), III (intron 1, exon 2, intron 2), IV (intron 2, exon 3, intron 3), V (intron 3, exon 4, intron 4), VI (intron 4, exon 5, 3'-UTR) and VII (exon 5, 3'-UTR) of "Serra da Estrela"*
o*GH* gene separated by native PAGE under non-denaturing conditions. The horizontal line is a schematic representation of the o*GH* gene. Exons are represented by black boxes and UTRs by striped boxes. Boundaries of these fragments are indicated in base pairs according to the published o*GH* gene sequence (exons following Orian *et al*., 1988; GenBank accession number X12546; 3'-UTR following UTR accession number: 3OAR000234). Different PCR-SSCP patterns within each fragment were randomly identified with a capital letter.

Table IV-1. *SSCP patterns found at the o*GH *fragments I, II, III and IV, the corresponding genotypes (in italic figures) and frequencies (%) in "Serra da Estrela" sheep population (SE) and in each of the seven flocks (FL1 to FL7).*

Fragment	SSCP pattern	Genotype*	SE (523)	Flock						
				FL1 (58)	FL2 (71)	FL3 (71)	FL4 (73)	FL5 (116)	FL6 (82)	FL7 (52)
(I_N)/(I_Z)**	A	(*1/1*)/(na)	91.88	98.28	66.67	88.57	91.55	97.41	98.77	100.00
	B	(*2/3*)/(na)	6.77	0.00	28.99	11.43	7.04	1.72	0.00	0.00
	C,D	nd	1.35	1.72	4.35	0.00	1.41	0.86	1.23	0.00
(II_N)/(II_Z)	A	(*1/1*)/(*1/1*)	93.12	87.93	95.77	100.00	65.75	100.00	98.78	100.00
	B	*(1/2)/(1/1)* or (*2/2*)/(*1/1*)	6.88	12.07	4.23	0.00	34.25	0.00	1.22	0.00
(III_N)/(III_Z)	A	(*1/4*)/(*3/3*)	22.41	29.31	26.76	1.41	27.40	18.97	34.57	19.23
	B	(*1/5*)/(*1/1*)	18.01	6.90	23.94	46.48	16.44	7.76	13.58	15.38
	C	(*1/1*)/(*1/1*)	16.09	10.34	15.49	11.27	23.29	14.66	19.75	17.31
	D	(*1/1*)/(*2/3*)	11.11	13.79	1.41	1.41	6.85	27.59	6.17	11.54
	E	(*1/1*)/(*1/1*)	7.47	8.62	14.08	2.82	16.44	1.72	1.23	13.46
	F	(*1/1*)/(*2/2*)	4.98	3.45	0.00	9.86	2.74	8.62	1.23	7.69
	G	(*1/1*)/(*2/4*)	4.41	5.17	2.82	4.23	4.11	5.17	7.41	0.00
	H	(*1/1*)/(*1/5*)	3.64	1.72	8.45	4.23	2.74	1.72	3.70	3.85
	I	(*1/3*)/(*2/2*)	3.26	3.45	1.41	4.23	0.00	6.90	1.23	3.85
	J	(*1/2*)/(*1/2*)	2.30	1.72	0.00	7.04	0.00	2.59	1.23	3.85
	K	(*1/1*)/(*1/4*)	1.92	0.00	0.00	2.82	0.00	2.59	4.94	1.92
	L	(*1/6*)/(*4/4*)	1.72	5.17	2.82	1.41	0.00	0.00	2.47	1.92
	M	(*1/1*)/(nd)	1.53	6.90	1.41	0.00	0.00	1.72	1.23	0.00
	O	(*1/1*)/(*-/-*)	0.38	0.00	0.00	1.41	0.00	0.00	0.00	0.00
	N,P,Q	nd	0.77	3.45	1.41	1.41	0.00	0.00	1.23	0.00
(IV_N)/(IV_Z)	A	(*1/2*)/(*1/1*)	57.55	74.14	61.97	22.54	87.67	48.28	70.73	38.46
	B	(*1/1*)/(*1/2*)	19.69	3.45	28.17	46.48	8.22	17.24	10.98	25.00
	C	(*1/1*)/(*1/3*)	8.60	1.72	0.00	19.72	1.37	12.07	6.10	19.23
	D	(*1/2*)/(*1/3*)	5.54	12.07	1.41	0.00	1.37	12.07	2.44	7.69
	E	(*1/3*)/(*3/3*)	4.59	3.45	2.82	5.63	0.00	9.48	0.00	9.62
	F	(*1/4*)/(*3/4*)	2.10	5.17	2.82	2.82	1.37	0.00	3.66	0.00
	G	(*1/1*)/(*5/5*)	1.72	0.00	2.82	1.41	0.00	0.86	6.10	0.00
	H	(*1/2*)/(*-/-*)	0.38	0.00	1.41	1.41	0.00	0.00	0.00	0.00

* - Genotypes were determined by copy specific fragment sequencing (for individual fragment allele details see **Table IV-4**); *(1/1)/(1/1) – GH2-N*/*GH2-Z* homozygous genotype at both oGH copies; *(1/2)/(1/2) – GH2-N/GH2-Z* heterozygous genotype at both oGH copies; *(1/2)/(1/1) - GH2-N*/*GH2-Z* heterozygous genotype at *GH2-N* copy; *(1/1)/(1/2) – GH2-N/GH2-Z* heterozygous genotype at *GH2-Z* copy; *(1/1)/(-/-)* – GH1 homozygous genotype (*GH2-Z* copy absent) etc.; (n) - number of animals; na – not analysed by SSCP; nd - genotype not determined.
** - SSCP analysis performed either at the *GH1* copy or at the *GH2-N* copy of the o*GH* gene

Table IV-2. *SSCP patterns found at the oGH fragments V, VI and VII, the corresponding genotypes (in italic figures) and frequencies (%) in "Serra da Estrela" sheep population (SE) and in each of the seven flocks (FL1 to FL7).*

Fragment	SSCP pattern	Genotype*	SE (523)	Flock						
				FL1 (58)	FL2 (71)	FL3 (71)	FL4 (73)	FL5 (116)	FL6 (82)	FL7 (52)
(V_N)/(V_Z)	A	(*1/1*)/(*1/1*) or (*1/1*)/(*-/-*)	84.70	58.62	77.46	91.55	75.34	93.97	93.90	92.31
	B	(*1/1*)/(*1/1*)	15.30	41.38	22.54	8.45	24.66	6.03	6.10	7.69
(VI_N)/(VI_Z)	A	(*1/1*)/(*1/2*)	48.18	15.52	64.79	84.51	35.62	41.38	43.90	51.92
	B	(*1/1*)/(*1/1*) or (*1/1*)/(*-/-*)	31.74	29.31	14.08	7.04	15.07	53.45	50.00	38.46
	C	(*2/3*)/(*1/2*)	13.58	36.21	21.13	8.45	19.18	5.17	4.88	9.62
	D	(*4/5*)/(*1/3*)	5.35	13.79	0.00	0.00	27.40	0.00	0.00	0.00
	E	(*6/7*)/(*3/3*)	1.15	5.17	0.00	0.00	2.74	0.00	1.22	0.00
(VII_N)/(VII_Z)	A	(*1/1*)/(*1/3*)	36.82	13.79	21.43	57.35	13.70	64.91	35.80	28.85
	B	(*1/5*)/(*2/3*)	25.19	15.52	45.71	32.35	31.51	6.14	19.75	40.38
	C	(*1/1*)/(*1/1*)	12.79	13.79	10.00	0.00	5.48	16.67	25.93	13.46
	D	(*2/2*)/(*2/3*)	9.69	24.14	14.29	7.35	10.96	4.39	3.70	9.62
	E	(*3/3*)/(*4/4*)	5.81	10.34	0.00	0.00	5.48	7.02	11.11	5.77
	F	(*6/7*)/(*3/3*)	5.43	17.24	7.14	0.00	10.96	0.88	3.70	1.92
	G	(*2/2*)/(*2/4*)	4.26	5.17	1.43	2.94	21.92	0.00	0.00	0.00

* - Genotypes were determined by copy specific fragment sequencing (for individual fragment allele details see **Table IV-4**); *(1/1)/(1/1) – GH2-N/GH2-Z* homozygous genotype at both oGH copies; *(1/2)/(1/2) – GH2-N/GH2-Z* heterozygous genotype at both oGH copies; *(1/2)/(1/1) - GH2-N/GH2-Z* heterozygous genotype at *GH2-N* copy; *(1/1)/(1/2) – GH2-N/GH2-Z* heterozygous genotype at *GH2-Z* copy; *(1/1)/(-/-)* – GH1 homozygous genotype (*GH2-Z* copy absent) etc.; (n) - number of animals.

In fragment (I_N)/(I_Z), the SSCP pattern A was found to be the most frequent in all flocks (**Table IV-1**). SSCP pattern B, the second most frequent in the overall population (6.77%), is present in 15.82% of the black variety FL2, FL3 and FL4 flocks, is absent in the white variety FL1, FL6 and FL7 flocks or rare (FL5 – 1.72%). Patterns C and D are absent in FL3 and FL7 flocks (high milk yielding flocks).

SSCP pattern A of fragment (II_N)/(II_Z) is the most frequent in all flocks. SSCP pattern B was not found in FL3, FL5 and FL7 flocks, being quite rare in FL6 (1.23%) and having some expression in low milk yielding FL1 (12.07%) and FL4 (34.25%) flocks.

o*GH* fragment (III_N)/(III_Z) is the most polymorphic with eleven SSCP patterns or more, in all flocks except in FL4, which is the less polymorphic flock with eight SSCP patterns (**Table IV-1**). SSCP pattern A is the most frequent in all but in FL3 and FL5. The most frequent SSCP pattern is pattern B in FL3 (46.48%) and pattern D in FL5 (27.59%). Some

SSCP patterns are relatively frequent in particular flocks, and were found at low frequencies or not found at all in other flocks, e.g., pattern A with 1.41% in FL3 and with 18 to 35% in the other flocks; pattern D with 1.41% in FL2 and FL3 and with 27.59% in FL5; pattern O was only found in FL3, but at a very low frequency (1.41%).

In fragment $(IV_N)/(IV_Z)$, the SSCP pattern A was found to be the most frequent in all flocks except in FL3. The most frequent SSCP pattern in FL3 is pattern B (46.48%). The frequency distribution between flocks was found to be quite wide in patterns A (ranging from 22.54% in FL3 to 87.67% in FL4) and B (ranging from 3.45% in FL1 to 46.48% in FL3) (**Table IV-1**).

In fragment $(V_N)/(V_Z)$, the SSCP pattern A was found to be the most frequent in all flocks (ranging from 58.62% in FL1 to almost 94% in FL5 and FL6). Pattern B, the less frequent one, was found more often in the low milk yielding FL1 (41.38%) and F4 (24.66%) flocks and also in FL2 (22.54%), a medium milk yielding flock (**Table IV-2).**

In fragment $(VI_N)/(VI_Z)$, the most frequent SSCP patterns are pattern A in FL2 (64.79%), FL3 (84.51%), FL4 (35.62%) and FL7 (51.92%); pattern B in FL5 (53.45%) and FL6 (50.00%); and pattern C in FL1 (36.21%). Pattern D was only found in the low milk yielding flocks FL1 (13.79%) and FL4 (27.40%), being FL4 the second more frequent pattern.

In fragment $(VII_N)/(VII_Z)$, the most frequent SSCP patterns are pattern A in FL3 (57.35%), FL5 (64.91%) and FL6 (35.80%); pattern B in FL2 (45.71%), FL4 (31.51%) and FL7 (40.38%); and pattern D in FL1 (24.14%).

FL7 flock presents a smaller number of SSCP patterns in all but in fragment $(V_N)/(V_Z)$ (**Table IV-1** and **Table IV-2**).

IV.2.2 Hardy-Weinberg equilibrium test

HWE tests were performed in each flock and in the overall population considering each of the o*GH* copy gene fragments as an individual locus and SSCP alleles as the loci' alleles, (**Table IV-3**). Tests were also made to assess heterozygote deficit or excess, as heterozygote disequilibrium could reflect the influence of factors such as selection, inbreeding or the existence of null alleles.

HWE was found in fragment II_N and III_N in all flocks and in fragment IV_N in FL3 and fragment IV_Z in FL4. The total population was not in HWE ($\chi^2=\infty$; df=84; $P<0.001$).

Heterozygote deficit was found in fragments I_N in flock FL5; in fragment VII_N in all but FL3; in fragment III_Z in all flocks; in fragment IV_Z in FL2, FL3, FL5 and FL6; in fragment VI_Z in FL6 and in fragment VII_Z in all flocks. Heterozygote excess was found in fragment III_N in all flocks; in fragment IV_N in all but FL3; and in fragment VI_Z in FL2, FL3, FL4, FL5 and FL7.

Table IV-3. *Hardy-Weinberg equilibrium, heterozygote deficit and excess tests' significance levels for the o*GH *copy gene fragments I_N to IV_N, VI_N, VII_N, III_Z, IV_Z, VI_Z and VII_Z.*

Flock	*P-value*	I_N	II_N	III_N	III_Z	IV_N	IV_Z	VI_N	VI_Z	VII_N	VII_Z
FL1	P_{HWE}	-	ns	ns	***	***	**	***	***	***	***
	P_D	-	ns	ns	***	ns	†	ns	ns	***	***
	P_E	-	ns	**	ns	***	ns	ns	ns	ns	ns
FL2	P_{HWE}	***	ns	ns	***	***	***	***	***	***	***
	P_D	ns	ns	ns	***	ns	***	ns	ns	***	***
	P_E	ns	ns	**	ns	***	ns	ns	***	ns	ns
FL3	P_{HWE}	***	-	†	***	ns	***	***	***	***	***
	P_D	ns	-	ns	***	ns	*	ns	ns	ns	**
	P_E	ns	-	***	ns	†	ns	ns	***	ns	ns
FL4	P_{HWE}	***	ns	ns	***	***	†	***	***	***	***
	P_D	ns	ns	ns	***	ns	ns	ns	ns	***	***
	P_E	ns	†	**	ns	***	ns	ns	***	ns	ns
FL5	P_{HWE}	***	-	ns	***	***	***	***	***	***	***
	P_D	**	-	ns	**	ns	***	ns	ns	**	***
	P_E	ns	-	**	ns	***	ns	ns	***	ns	ns
FL6	P_{HWE}	-	-	ns	***	***	***	***	***	***	***
	P_D	-	-	ns	***	ns	**	ns	**	**	***
	P_E	-	-	***	ns	***	ns	ns	ns	ns	ns
FL7	P_{HWE}	-	-	ns	***	*	*	***	**	***	***
	P_D	-	-	ns	***	ns	ns	ns	ns	*	***
	P_E	-	-	*	ns	**	ns	ns	***	ns	ns
ALL[1]	χ^2	223.9	4.4	15.8	∞	221.0	∞	∞	234.7	∞	628.3
	Df	8	6	14	14	14	14	14	14	14	14
	Sign.	***	ns	ns	***	***	***	***	***	***	***

[1] – Detemined by Fisher's method for the seven flocks; P_{HWE} – significance level for HWE test; P_D - significance level for heterozygote deficit; - P_E - significance level for heterozygote excess.
ns – P>0.10; † - P>0.05; * - P<0.05; ** - P<0.01; *** - P<0.001.

IV.2.3 Sequencing of the inter-copy region and separate PCR-SSCP fragment analysis of each o*GH* gene copy

The duplication of the *GH* gene in *Ovis aries* causes the PCR-SSCP analysis to be complex. A minimum of one and a maximum of four alleles can be differentiated by the SSCP analysis depending on the copy number genotype and on the zygotic condition (homo or hetero) of the animal for the analysed DNA fragment. In order to specifically assign the SSCP bands to

each copy the following strategy was followed. The inter-copy region was PCR amplified, cloned and sequenced (GenBank accession number: DQ238053). Sequence analysis disclosed major differences in the 5' regions of the o*GH* copies (**Figure IV-6**) allowing the design of primers for the specific amplification of each copy.

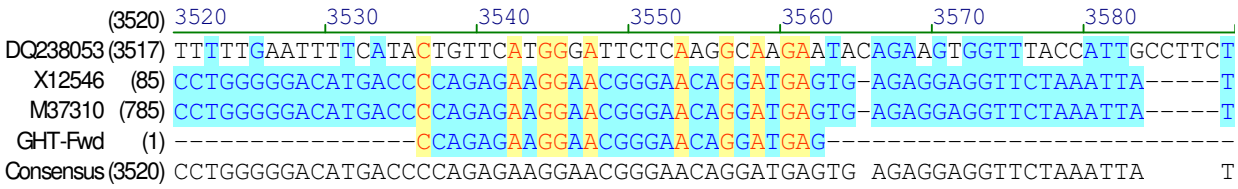

Figure IV-6. *Alignment of the sequence of a fragment of the inter-copy region DQ238053 (our results) containing the 5'-UTR of the* GH2-Z *copy with previously published sequences of fragments containing the 5'-UTR of the* GH2-N *copy (M37310; Byrne* et al.*, 1987) and* GH1 *copy (X12546; Orian* et al.*, 1988) and with primer GHT-Fwd specific for* GH1 *copy (or* GH2-N*) amplification.*
Note: Aligned sequences present any non-similar (N), identical (N) or conservative (N) nucleotides in each position.

The products of these specific amplifications were used as templates for separate PCR-SSCP fragment analysis. Superimposition of the SSSP patterns from each copy generated the patterns obtained with the non-specific copy fragment amplification performed on genomic DNA (**Figure IV-7**).

Figure IV-7. *PCR-SSCP analysis of the fragment IV of the o*GH *gene.*
IV – PCR-SSCP pattern C of the fragment IV [(1/1)/(1/3) genotype (for individual fragment allele details see **Table IV-4**)]: amplification from genomic DNA was not copy specific.
IV_N – PCR-SSCP pattern B of the fragment IV_N, a DNA specifically PCR amplified from the *GH2-N* copy was used as a template for fragment IV amplification.
IV_Z – PCR-SSCP pattern B of the fragment IV_Z, a DNA specifically PCR amplified from the *GH2-Z* copy was used as a template for fragment IV amplification.

For instance, SSCP pattern B of fragment VI has two bands suggesting that animals exhibiting this pattern are homozygous for the copy number and/or for the exon 5 containing fragment (**Figure IV-5**, **Table IV-2**). This was confirmed by Southern blotting analysis which showed that one animal presenting this pattern had the *Gh1*/*Gh1* genotype. In addition, sequencing showed that the remaining animals with *Gh2*/*Gh2* genotype were homozygous for fragment VI. The more complex pattern C in fragment IV exhibits six bands suggesting that

animals presenting this pattern have the *Gh2*/*Gh2* genotype and are heterozygous for the fragment IV in one copy and homozygous in the other, or alternatively have the *Gh1*/*Gh2* genotype and are heterozygous for the *GH2-N* copy. Southern blotting analysis and sequencing showed that the first hypothesis applies (**Table IV-2** and **Figure IV-7**).

IV.2.4 Molecular characterization of the SSCP patterns

Twenty sheep carrying each of the SSCP patterns shown in **Figure IV-5** were selected and genotyped for the copy number. One of these animals had the *Gh1*/*Gh1* genotype, two the *Gh1*/*Gh2* genotype and 17 the *Gh2*/*Gh2* genotype. The nucleotide sequence of each *oGH* gene copy fragment was established for the selected animals. SSCP alleles at each fragment were molecularly characterized and denoted by an italic figure (**Table IV-1** and **Table IV-4**): e.g., allele *1* of fragment II_N [at *GH2-N* (*or GH1*) copy] carries bases T_{301} (T at position 301, according to sequence GenBank accession number X12546; Orian *et al*., 1988) and G_{305}; allele *1* of fragment III_Z (at *GH2-Z* copy) carries bases G_{644}, C_{649}, C_{668}, C_{704} and G_{712}. For each fragment's SSCP patterns, a fragment genotype was thus established (**Table IV-1** and **Table IV-2**).

A total of 2055 bp were sequenced at the *GH2-N* (or *GH1*) copy and of 2100 bp at *GH2-Z* copy in each of the selected animals. Twenty-four polymorphic sites were found at the *GH2-N* (or *GH1*) copy and 14 at the *GH2-Z* copy. Some of these polymorphisms were previously reported (**Table IV-4**). Comparing with the *GH1* sequence firstly reported by Orian *et al*. (1988) the *GH2-N* (or *GH1*) copy presented a total of 16 transitions, six transversions and two insertions/deletions of one nucleotide each. Comparing with the *GH2-Z* sequence reported by Ofir and Gootwine (1997) the *GH2-Z* copy showed a total of 11 transitions and three transversions. Nucleotide polymorphisms were found in all of the studied fragments of the *GH2-N* (or *GH1*) copy but in fragment V_N and in fragments III_Z, IV_Z, VI_Z and VII_Z of the *GH2-Z* copy. Although two SCCP patterns were found in the fragments V_N and V_Z no polymorphisms were identified.

The polymorphic sites were distributed as follows at the *GH2-N* (or *GH1*) copy: two in fragment I_N, two in fragment II_N, seven in fragment III_N, three in fragment IV_N, four in fragment VI_N and seven in fragment VII_N; at the *GH2-Z* copy: five in fragment III_Z, five in fragment I_Z, two in fragment V_Z and two in fragment VI_Z. The *GH2-N* copy was found to be more polymorphic than the *GH2-Z* copy. The number of alleles ranged from two in fragment I_N to seven in fragment V_N and fragment VI_N.

Table IV-4. *Nucleotide sequence characterisation of SSCP alleles of each amplified fragment at the GH2-N (or GH1) and GH2-Z copies.*

oGH Fragment (Type)	*GH2-N* (or *GH1*) alleles[1] Site [3]	*1*	*2*	*3*	*4*	*5*	*6*	*7*	oGH Fragment (Type)	*GH2-Z* alleles [2] Site [3]	*1*	*2*	*3*	*4*	*5*
I_N	$5UN_{288}$	C	T	T											
(5'-UTR, E1)	$5UN_{293}$	C	C	A											
II_N	$E1N_{301}$	T	A												
(5'-UTR, E1, I1)	$E1N_{305}$	G	A												
III_N	$E2N_{597}$	T	T	C	T	T	T		III_Z	$E2Z_{644}$	G	G	G	A	G
(I1, E2, I2)	$E2N_{651}$	C	C	C	C	C	T		(I1, E2, I2)	$E2Z_{649}$	C	C	C	C	G
	$E2N_{666}$	C	T	C	C	C	C			$E2Z_{668}$	C	T	C	C	C
	$E2N_{708}$	A	A	A	A	G	A			$E2Z_{704}$	C	C	C	C	G
	$E2N_{717}$	C	C	C	C	C	T			$E2Z_{712}$	G	A	A**	A	G
	$E2N_{733}$	A	A	A	G	A	A								
	$E2N_{738}$	T	T	C	T	T	T								
IV_N	$E3N_{973}$	A	A	T	A				IV_Z	$E3Z_{1035}$	T	C	T	C	C
(I2, E3, I3)	$E3N_{1024}$	T	T	C	C				(I2, E3, I3)	$E3Z_{1047}$	A	A	G	G	G
	$E3N_{1047}$	A	G*	A	A					$E3Z_{1057}$	A	G	A	G	G
										$E3Z_{1062}$	G	G	G	G	C
										$I3Z_{1100}$	A	G	G	G	G
VI_N	$E5N_{1872}$	G	A	G	G	G	G	G	VI_Z	$E5Z_{1852}$	G	G	A		
(I4, E5, 3'-UTR)	$E5N_{1938}$	C	C	C	C	C	C	T	(I4, E5, 3'-UTR)	$3UZ_{1965}$	T**	C	C		
	$3UN_{1980}$	-	T	T	C	T	T	T							
	$3UN_{2024}$	-	-	-	-	-	T	T							
VII_N	$3UN_{1980}$	-	T	T	C	-	-	-	VII_Z	$3UZ_{1965}$	T	T	C	C	
(E5, 3'-UTR)	$3UN_{2024}$	-	-	T	-	-	-	-	(E5, 3'-UTR)	$3UZ_{2030}$	T	C	C	T	
	$3UN_{2036}$	A	A	A	A	A	A	G							
	$3UN_{2039}$	C	C	C	C	T	C	C							
	$3UN_{2050}$	C	C	C	C	G	C	C							
	$3UN_{2059}$	G	G	G	G	G	T	T							
	$3UN_{2069}$	G	G	G	G	G	C	C							

[1] Underlined: variants from the *GH1* copy sequence reported by Orian *et al*. (1988; GenBank accession number X12546); * Australian Merino breed *GH* sequence reported by Byrne *et al*. (1987; GenBank accession number M37310).

[2] Underlined: variants from the Awassi breed *GH2-Z* copy sequence reported by Ofir and Gootwine (1997; GenBank accession number AF002124 to AF002129); ** Romanov breed *GH2-Z* copy sequence reported by Ofir and Gootwine (1997; GenBank accession no. AF002118 and AF002120).

[3] Site: U – UTR; E – Exon; I – intron; *N* – *GH2-N* (or *GH1*) copy; *Z* – *GH2-Z* copy; e.g., $5UN_{288}$ – 5'-UTR at *GH2-N* (or *GH1*) copy, position 288 (According to the published *o*GH gene sequence (Orian *et al*. 1988; GenBank accession no. X12546).

Table IV-5. *Polymorphisms found in the coding regions of the o*GH *gene copies, predicted amino acid changes and protein variants.*

oGH	*GH1* or *GH2-N*				Variants					*GH2-Z*				Variants		
Fragment	Site*	nt	aa no.	aa	a	b	c	d	e	Site*	nt	aa no.	aa	a	b	c
III	E2N_{597}	T→C	-16	Leu→Pro	L	L	P	L	L	E2Z_{644}	G→A	-1	Gly	G	G	G
(E2)	E2N_{651}	C→T	3	Pro→Leu	P	P	P	P	L	E2Z_{649}	C→G	2	Phe→Leu	F	F	L
	E2N_{666}	C→T	8	Ser→Phe	S	F	S	S	S	E2Z_{668}	C→T	9	Arg→Cys	R	C	R
	E2N_{708}	A→G	22	His→Arg	H	H	H	R	H	E2Z_{704}	C→G	21	Leu→Val	L	L	V
	E2N_{717}	C→T	25	Ala→Val	A	A	A	A	V	E2Z_{712}	G→A	23	Gln	G	G	G
	E2N_{733}	A→G	30	Lys	K	K	K	K	K							
	E2N_{738}	T→C	32	Phe→Ser	F	F	S	F	F							
IV	E3N_{973}	A→T	35	Thr→Ser	T	S	T			E3Z_{1035}	T→C	55	Ser	S	S	S
(E3)	E3N_{1024}	T→C	52	Phe→Leu	F	L	L			E3Z_{1047}	A→G	59	Pro	P	P	P
	E3N_{1047}	A→G	59	Pro	P	P	P			E3Z_{1057}	A→G	63	Ser→Gly	S	G	G
										E3Z_{1062}	G→C	64	Asn→Lys	K	K	N
VI	E5N_{1872}	G→A	166	Arg	R					E5Z_{1852}	G→A	160	Gly→Ser	G	S	
(E5)	E5N_{1938}	C→T	188	Ser	S											

* According to the published oGH gene sequence (Orian *et al*., 1988; GenBank accession number X12546). E – exon.
nt – nucleotide; aa no. – amino acid number of the mature protein (considering N-terminal alanine as amino acid 1 which corresponds to amino acid 27 of the published oGH sequence with GenPept accession number CAA31063) (Orian *et al*., 1988).

IV.2.4.1 oGH haplotypes

A total of 86 haplotypes at the *GH2-N* copy and 32 at the *GH2-Z* copy were inferred from SSCP allele composition, and the DNA sequences of the SSCP alleles shown in **Table IV-4**. Haplotypes were constructed by joining together, separated by a comma, the alleles of the analysed fragments, e.g. a *GH2-N* haplotype composed by allele *1* at fragments I to VII is denominated (*1,1,1,1,1,1,1*). Whenever phase was determine, the haplotypes of a genotype were separated by /, e.g. the N1 genotype of the *GH2-N* copy is *(1,1,1,1,1,1,1)/(1,1,1,2,1,1,1).* Fragment genotypes were separated by a comma whenever phase could not be determined. For example, an animal with genotype (*1/1*) at fragments I, II and III, genotype (*1/2*) at fragment IV, genotype (*1/1*) at fragments V and VI, and genotype (*1/5*) at fragment VII has a genotype denominated *(1/1,1/1,1/1,1/2,1/1,1/1,1/5).* The three most frequent haplotypes were *Hn1* (*1,1,1,1,1,1,1* - 42.67%), *Hn2* (*1,1,1,1,1,1,5* - 11.60%) and *Hn3* (*1,1,1,2,1,1,1* - 5.04%) for the *GH2-N* copy; and *Hz1* (*1,1,1,1,2,3* - 14.74%), *Hz2* (*1,2,3,1,1,2* - 11.16%), and *Hz3* (*1,1,1,1,1,1* - 7.87%), *Hz4* (*1,1,1,1,1,2* - 7.87%) and *Hz5* (*1,3,1,1,1,1* - 7.87%) for the *GH2-Z* copy.

IV.2.4.2 oGH protein variants

Synonymous and non-synonymous substitutions were found in the exons 2, 3 and 5 of the *oGH* copies (**Table IV-5**). Eight non-synonymous substitutions were predicted in the *GH2-N* copy and six in the *GH2-Z* copy.

Eight GH2-N protein variants (A-F, H and I) are predicted considering non-synonymous substitutions. The variant A (V_NA) of the GH2-N copy with amino acid partial sequence $L^{-16},P^{3},S^{8},H^{22},A^{25},F^{32},T^{35},F^{52}$ corresponds to the published oGH sequence with GenPept accession number CAA31063 (Orian *et al*., 1988). The remaining variants differ from A at the following residues: var. B - S^{35},L^{52}; var. C - L^{52}; var. D - R^{22}; var. E - P^{-16},S^{32}; var. F - F^{8}; var. H – $P^{-16},S^{32},S^{35},L^{52}$; var. I - L^{3},V^{25}. Synonymous substitutions were found at residues K^{30} in exon 2, P^{59} in exon 3, R^{166} and S^{188} in exon 5. Protein variants A (91.98%) and D (4.69%) were found to be the most frequent ones at the GH2-N copy (**Table IV-6**).

Ten GH2-Z protein variants (A-J) are predicted considering non-synonymous substitutions. The variant A (V_ZA) of the GH2-Z copy with amino acid partial sequence $F^{2},R^{9},L^{21},S^{63},K^{64},G^{160}$ corresponds to the published oGH sequence number Q95205 (Lacroix *et al*., 1996). The remaining variants differ from A at the following amino acids: var. B - C^{9}; var. C - S^{160}; var. D - C^{9},G^{63}; var. E - G^{63}; var. F - L^{2},V^{21},G^{63}; var. G - C^{9},S^{160}; var. H - L^{2},V^{21},S^{160}; var. I - L^{2},V^{21} and var. J – G^{63},N^{64}. Synonymous substitutions were found at residues G^{-1} and

G^{23} in exon 2, and S^{55} and P^{59} in exon 3. Protein variants A (71.89%), B (13.29%) and E (7.68%) were found to be the most frequent ones at the GH2-Z copy (**Table IV-6**).

Table IV-6. *Predicted protein variants' frequencies (%) at the GH2-N and GH2-Z copies.*

V_N	GH2-N	(%)	V_Z	GH2-Z	(%)
A	$\mathbf{L^{-16},P^{3},S^{8},H^{22},A^{25},F^{32},T^{35},F^{52}}$	91.98	**A**	$\mathbf{F^{2},R^{9},L^{21},S^{63},K^{64},G^{160}}$	71.89
B	$L^{-16},P^{3},S^{8},H^{22},A^{25},F^{32},\mathbf{S^{35},L^{52}}$	0.69	**B**	$F^{2},\mathbf{C^{9}},L^{21},S^{63},K^{64},G^{160}$	13.29
C	$L^{-16},P^{3},S^{8},H^{22},A^{25},F^{32},T^{35},\mathbf{L^{52}}$	0.57	**C**	$F^{2},R^{9},L^{21},S^{63},K^{64},\mathbf{S^{160}}$	3.90
D	$L^{-16},P^{3},S^{8},\mathbf{R^{22}},A^{25},F^{32},T^{35},F^{52}$	4.69	**D**	$F^{2},\mathbf{C^{9}},L^{21},\mathbf{G^{63}},K^{64},G^{160}$	0.86
E	$\mathbf{P^{-16}},P^{3},S^{8},H^{22},A^{25},\mathbf{S^{32}},T^{35},F^{52}$	0.46	**E**	$F^{2},R^{9},L^{21},\mathbf{G^{63}},K^{64},G^{160}$	7.68
F	$L^{-16},P^{3},\mathbf{F^{8}},H^{22},A^{25},F^{32},T^{35},F^{52}$	0.58	**F**	$\mathbf{L^{2}},R^{9},\mathbf{V^{21}},\mathbf{G^{63}},K^{64},G^{160}$	0.20
H	$\mathbf{P^{-16}},P^{3},S^{8},H^{22},A^{25},\mathbf{S^{32},S^{35},L^{52}}$	0.57	**G**	$F^{2},\mathbf{C^{9}},L^{21},S^{63},K^{64},\mathbf{S^{160}}$	0.40
I	$L^{-16},\mathbf{L^{3}},S^{8},H^{22},\mathbf{V^{25}},F^{32},T^{35},F^{52}$	0.46	**H**	$\mathbf{L^{2}},R^{9},\mathbf{V^{21}},S^{63},K^{64},\mathbf{S^{160}}$	0.20
			I	$\mathbf{L^{2}},R^{9},\mathbf{V^{21}},S^{63},K^{64},G^{160}$	1.49
			J	$F^{2},R^{9},L^{21},\mathbf{G^{63}},\mathbf{N^{64}},G^{160}$	0.09

V_N – predicted protein variant at the GH2-N copy; V_Z – predicted protein variant at the GH2-Z copy.

In all the genotyped sheep exhibiting the two *oGH* copies (seventeen *Gh2*/*Gh2* and two *Gh1*/*Gh2* animals) two amino acid changes were always found between *GH2-N* and *GH2-Z* copies: aa-7 (P → L) and aa9 (G → R or C). Aa63 differs also between copies (G → S), except in animals carrying V_ZD, V_ZE, V_ZF and V_ZJ protein variants as they present a G^{63} in both copies.

IV.2.5 Statistical analysis

IV.2.5.1 Milk yield

The influence of oGH copies genotypes and phenotypes on milk yield adjusted to 150 lactation days was statistical analysed as described in **section III.6.2**. Data concerning 1704 valid lactations from 513 ewes' official milk records between 1996 and 2000 were used in the univarieted analysis. The mean milk yield adjusted to 150 lactation days (± SD) was 159.5 ± 72.2 l.

The statistical analyses were performed considering the *GH2-N* and *GH2-Z* copies genotypes (**Table IV-7** and **Table IV-8**) and phenotypes (**Table IV-10** and **Table IV-11**) taken separately or associated (**Table IV-9** and **Table IV-12**) in the overall population and also by flock. Contrasts were performed considering the differences of milk yield adjusted to 150 lactation days between the different genotypes or phenotypes and the most frequent homozygous genotype or phenotypes (whenever it was possible) in each o*GH* gene copy or associated *GH2-N* and *GH2-Z* genotype or phenotypes. Genotypes and phenotypes with frequencies lower than 2% were excluded from the analysis. The different genotypes and phenotypes found in the *GH2-N* and *GH2-Z* copies were found to have significant influence on milk yield in "Serra da Estrela" ewes ($P<0.05$). The analyses of the influence of o*GH* genotypes and phenotypes upon milk production within each flock showed that they influence milk yield differently depending presumably on flocks' management, namely at nutritional level, and on particular environmental factors of the regions where animals are reared.

Within *GH2-N* genotypes a milk yield differential of 21.4 ± 5.9 l/150 d ($P<0.01$) was found between the most (N7) and the least (N5) productive one (**Table IV-7**). The ewes with N7 genotype produced respectively 6.3 ± 5.3 l/150 d and 10.5 ± 5.6 l/150 d more milk than ewes with genotypes N1 and N2 which were the most abundant in the studied population (13.0% each), however this values were not statistically significant ($P>0.05$). By flock, the major significant differences found in milk yield of animals with different GH2-N genotypes were as follows: N11 ewes from FL2 yielded 27.2 ± 11.9 l/150 d more milk than N1 ewes ($P<0.05$); N1 ewes from FL3 yielded 79.2 ± 12.9 l/150 d more milk than N8 ewes ($P<0.001$); N18 ewes from FL5 yielded 52.3 ± 16.3 l/150 d more milk than N10 ewes ($P<0.05$); N4, N7 and N8 ewes from FL6 yielded respectively 47.6 ± 9.3 l/150 d, 66.7 ± 11.8 l/150 d and 62.2 ± 11.9 l/150 d more milk than N2 ewes ($P<0.001$), with no significant differences in milk yield between them ($P>0.05$); N18 ewes from FL7 yielded 83.7 ± 16.5 l/150 d more milk than N2 ewes ($P<0.001$). No significant milk yield differences were found between *GH2-N* genotypes at FL1+FL4 flock. Increasing the number of N7 and N18 genotypes might contribute to the improvement of milk yield in "Serra da Estrela" flocks. Ewes with the N5 genotype, the worst in the total population, were not the worst in any particular flock; nevertheless, their milk yield was always found to be low. Ewes with the N2 genotype were found to be the lowest producing ones in FL6 and FL7 high producing flocks, but in the other flocks they were within the best milk producing animals.

Table IV-7. GH2-N *genotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent homozygous* GH2-N *genotype in all flocks but FL1+FL4 (deviation from the most frequent genotype within the flock).*

GH2-N		Freq.	**All flocks**	Freq.	**FL1+FL4**	Freq.	**FL2**	Freq.	**FL3**	Freq.	**FL5**	Freq.	**FL6**	Freq.	**FL7**
Genotypes		(%)	(N=1150)	(%)	(N=281)	(%)	(N=178)	(%)	(N=161)	(%)	(N=294)	(%)	(N=214)	(%)	(N=143)
N1	*(1,1,1,1,1,1,1)/(1,1,1,2,1,1,1)*	13.0	4.2 ± 4.2 bc	4.2	-6.2 ± 9.4	12.8	-13.9 ± 10.1 a	7.9	27.2 ± 12.4 d	33.2	2.5 ± 6.4 cd	12.3	24.6 ± 9.7 b	8.5	39.4 ± 14.5 bc
N2	*(1,1,1,1,1,1,1)/(1,1,1,1,1,1,1)*	13.0	0 bc			3.8	0 ab	13.9	0 c	19.0	0 bc	9.9	0 a	10.7	0 a
N3	*(1/1,1/1,1/5,1/1,1/1,1/1,2/4)*	8.4	-3.8 ± 5.0 ab	4.0	8.0 ± 9.9	16.1	-2.5 ± 9.8 ab	25.5	-46.5 ± 9.3 a	3.0	15.6 ± 13.5 cd			13.0	59.2 ± 13.0 cd
N4	*(1/1,1/1,1/4,1/2,1/1,1/1,1/1)*	8.2	4.3 ± 4.9 bc	5.7	4.4 ± 7.8	11.4	6.3 ± 10.2 ab			6.9	-2.0 ± 9.9 abcd	18.3	47.6 ± 9.3 c	6.2	11.0 ± 15.0 ab
N5	*(1/1,1/1,1/1,1/2,1/1,1/1,2/4)*	6.2	-10.9 ± 5.3 a	3.5	-5.8 ± 9.0							20.4	13.2 ± 9.6 ab	13.0	21.8 ± 12.5 ab
N6	*(1/1,1/1,1/1,1/2,1/1,2/3,1/5)*	5.4	-3.3 ± 5.7 ab	13.4	0	3.3	-6.2 ± 17.4 ab	3.2	-13.8 ± 16.0 bc	3.0	-10.8 ± 13.9 abc			7.9	23.7 ± 14.5 ab
N7	*(1/1,1/1,1/4,1/2,1/1,1/1,6/7)*	5.0	10.5 ± 5.6 c	7.0	11.6 ± 7.6					6.9	6.4 ± 9.6 cd	7.4	66.7 ± 11.8 c	7.9	40.3 ± 15.9 bc
N8	*(1,1,1,1,1,1,1)/(1,1,5,1,1,1,1)*	3.5	-4.2 ± 6.4 ab					11.6	-52.0 ± 11.1 a	4.5	-1.0 ± 11.7 abcd	7.0	62.2 ± 11.9 c		
N9	*(1/1,1/1,1/4,1/2,1/1,2/3,3/3)*	3.2	8.3 ± 6.6 bc	7.2	3.8 ± 7.1	5.2	0.8 ± 13.9 ab								
N10	*(1,1,1,1,1,1,1)/(1,1,2,1,1,1,1)*	2.2	-0.1 ± 7.3 abc					6.5	0.2 ± 12.1 cd	3.6	-28.5 ± 13.6 a				
N11	*(1/1,1/1,1/4,1/2,1/1,2/3,1/5)*	2.0	4.3 ± 7.9 abc			9.5	13.3 ± 10.2 b								
N12	*(2/3,1/1,1/1,1/2,1/1,1/1,2/4)*	1.9	7.2 ± 8.1 bc			10.9	0.6 ± 10.6 ab								
N13	*(1,1,1,1,1,1,2)/(1,1,1,1,1,1,4)*													9.0	57.3 ± 13.6 cd
N14	*(1/1,1/2,1/1,1/2,1/1,4/5,2/2)*			5.7	2.2 ± 7.9										
N15	*(1/1,1/2,1/1,1/2,1/1,4/5,2/4)*			6.0	4.0 ± 8.1										
N16	*(2,1,1,1,1,1,1)/(3,1,1,1,1,1,1)*							6.0	-30.8 ± 13.1 ab						
N17	*(2/3,1/1,1/5,1/1,1/1,1/1,2/4)*					8.5	0.5 ± 10.9 ab								
N18	*(1,1,1,1,1,1,1)/(1,1,1,3,1,1,1)*									4.5	23.8 ± 11.5 d			4.5	83.7 ± 16.5 d
N19	*(1,1,1,1,1,1,1)/(1,1,1,4,1,1,1)*			2.5	-8.9 ± 10.8										
N20	*(1/1,1/1,1/3,1/3,1/1,1/1,1/1)*									3.9	-21.7 ± 11.9 ab				
N21	*(1/1,1/1,1/4,1/2,1/1,1/1,2/2)*			4.0	-3.0 ± 9.2										
N22	*(1/1,1/2,1/1,1/2,1/1,6/7,3/3)*			3.0	6.2 ± 10.5										
N23	*(1/1,1/2,1/5,1/2,1/1,4/5,2/4)*			3.7	-4.4 ± 9.5										
N24	*(2/3,1/1,1/1,1/2,1/1,1/1,1/1)*					2.8	-16.5 ± 17.0 ab								

Note: Genotypes with less than 2% of the total lactations are not shown.

Genotype notation: e.g. N1 - seven phase genotype of the *GH2-N* copy composed of haplotypes *(1,1,1,1,1,1,1)* and *(1,1,1,2,1,1,1)* separated by /; individual fragment (I to VII) alleles are represented by an italic figure and are separated by a comma; N3 - *(1/1,1/1,1/5,1/1,1/1,1/1,2/4)* unphased genotype of the *GH2-N* with individual fragment (I to VII) genotypes separated by a comma. For individual fragment allele details see **Table IV-4**.

N = number of lactations.

a, b, c, d – values in the same column with different letters are significantly different (P<0.05).

Table IV-8. GH2-Z *genotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent homozygous* GH2-Z *genotype.*

GH2-Z		Freq.	**All flocks**	Freq.	**FL1+FL4**	Freq.	**FL2**	Freq.	**FL3**	Freq.	**FL5**	Freq.	**FL6**	Freq.	**FL7**
Genotypes		(%)	(N=1007)	(%)	(N=288)	(%)	(N=183)	(%)	(N=163)	(%)	(N=218)	(%)	(N=185)	(%)	(N=93)
Z1	*(1,1,1,1,1,2)/(1,1,1,1,2,2)*	5.1	8.0 ± 5.3 abcd	2.7	5.4 ± 10.0	6.9	-5.4 ± 12.0	3.8	64.4 ± 15.5 d	10.0	0 bcd	5.5	-21.8 ± 11.4 a		
Z2	*(1/1,1/1,1/1,1/1,1/2,2/3)*	13.3	0 a	14.1	0	18.0	0	9.5	0 b			21.8	0 a	19.2	0 a
Z3	*(1,1,1,1,1,4)/(1,1,1,1,3,4)*	2.2	7.2 ± 7.8 abcd	8.7	2.9 ± 6.6										
Z4	*(1/1,1/1,1/2,1/1,1/2,2/2)*	4.3	14.9 ± 5.7 bcd					15.2	-27.1 ± 11.2 a	4.8	20.1 ± 12.0 cd	3.8	52.1 ± 13.4 bc		
Z5	*(1/1,1/1,1/2,1/1,1/2,2/3)*	10.7	3.8 ± 4.4 ab	5.0	5.1 ± 8.9	25.3	0.9 ± 8.3	27.0	-28.8 ± 10.3 a	3.0	15.0 ± 14.3 bcd			15.8	34.1 ± 10.3 b
Z6	*(1/1,1/5,1/1,1/1,1/2,2/2)*	2.3	6.3 ± 7.3 abcd			7.8	-10.4 ± 12.4	5.2	23.5 ± 15.6 bc						
Z7	*(1/1,2/2,1/3,1/1,1/2,2/2)*	3.2	21.6 ± 6.7 d					10.0	30.2 ± 12.4 c	7.0	-8.7 ± 11.2 ab			4.5	2.1 ± 14.9 ab
Z8	*(1/1,2/3,1/1,1/1,1/2,2/3)*	2.1	0.9 ± 7.5 abc	5.0	-5.5 ± 7.8					3.3	-11.4 ± 14.4 abc				
Z9	*(1,3,1,1,1,1)/(1,3,1,1,1,1)*	6.1	2.0 ± 5.0 ab	3.5	-9.5 ± 9.5	11.1	10.6 ± 11.2					16.6	26.1 ± 8.0 b	5.1	5.3 ± 16.8 ab
Z10	*(1,3,1,1,1,3)/(1,3,1,1,1,3)*	5.0	16.2 ± 5.3 cd	5.9	5.6 ± 7.8					7.0	1.8 ± 11.2 bcd	7.3	49.6 ± 10.3 c	7.9	23.0 ± 13.6 ab
Z11	*(1/1,3/3,1/1,1/1,1/2,2/3)*	2.4	0.5 ± 6.9 abc			8.8	12.2 ± 11.2								
Z12	*(1,3,1,1,1,4)/(1,3,1,1,2,4)*	5.0	8.7 ± 5.3 abcd	12.1	0.4 ± 6.0	6.5	-0.6 ± 12.1								
Z13	*(1/1,1/1,1/1,1/1,1/3,2/3)*			7.4	0.3 ± 7.1										
Z14	*(1/1,1/2,1/3,1/1,1/2,2/2)*							6.6	12.6 ± 13.2 bc						
Z15	*(1,2,1,1,1,2)/(1,3,1,1,1,2)*									7.6	-2.1 ± 10.4 abcd				
Z16	*(1,1,1,1,3,4)/(1,1,1,1,3,4)*			4.5	0.2 ± 8.8										
Z17	*(1/1,2/4,3/4,1/1,1/1,2/2)*			2.5	-10.3 ± 10.6										
Z18	*(1/1,2/3,1/3,1/1,1/1,1/1)*									4.5	-2.5 ± 12.8 abcd				
Z19	*(1/1,2/3,1/3,1/1,1/1,2/2)*									4.5	-7.2 ± 12.9 abcd				
Z20	*(1,2,3,1,1,2)/(1,3,3,1,1,2)*									4.2	24.1 ± 13.0 d				
Z21	*(1,2,3,1,1,2)/(1,2,3,1,1,2)*									3.9	-26.5 ± 13.0 a				
Z22	*(1/1,2/4,1/2,1/1,1/2,2/2)*									3.6	12.1 ± 14.4 bcd				
Z23	*(1,3,1,1,1,2)/(1,3,1,1,1,2)*									2.4	-12 ± 14.6 abc				
Z24	*(1,2,1,1,1,1)/(1,3,1,1,1,1)*											4.8	0.9 ± 11.5 a		
Z25	*(1/1,2/4,1/3,1/1,1/2,2/2)*											4.2	-21.4 ± 14.1 a		

Note: Genotypes with less than 2% of the total lactations are not shown.

Genotype notation: e.g. Z1 - six phase genotype of the *GH2-Z* copy composed of haplotypes *(1,1,1,1,1,2)* and *(1,1,1,1,2,2)* separated by /; individual fragment (II to VII) alleles are represented by an italic figure and are separated by a comma; Z2 - *(1/1,1/1,1/1,1/1,1/2,2/3)* unphased genotype of the *GH2-Z* with individual fragment (II to VII) genotypes separated by a comma. For individual fragment allele details see **Table IV-4**.

N = number of lactations.

a, b, c, d – values in the same column with different letters are significantly different (P<0.05).

Within *GH2-Z* genotypes a milk yield differential of 21.6 ± 6.7 l/150 d (P<0.01) was found between the most (Z7) and the least (Z2) productive one (**Table IV-8**). The ewes with Z2 genotype were found to be, simultaneously, the most abundant and less productive in the studied population (13.3%). By flock, the major significant differences in milk yield of animals with different GH2-Z genotypes are as follows: Z1 ewes from FL3 yielded respectively 93.2 ± 13.9 l/150 d and 91.6 ± 14.7 l/150 d more milk than Z5 and Z4 ewes (P<0.001); Z20 ewes from FL5 yielded 50.6 ± 15.7 l/150 d more milk than Z21 ewes (P<0.01); Z10 ewes from FL6 yielded respectively 71.4 ± 13.5 l/150 d and 71.0 ± 15.9 l/150 d more milk than Z1 and Z25 ewes (P<0.001); and Z5 ewes from FL7 yielded 34.1 ± 10.3 l/150 d more milk than Z2 ewes (P<0.01). No significant milk yield differences were found between *GH2-Z* genotypes at FL1+FL4 and FL2 flocks. Some contradictory results arise from **Table IV-8**, namely between results from FL3 *versus* all the other flocks; i.e., ewes with genotype Z1 are the best producing ewes from FL3, but the worst from FL6; and similarly ewes with Z5 are the best producing ewes from the flock FL7, but are ones of the worst from FL3.

The effect of associated *GH2-N* and *GH2-Z* genotypes revealed a milk yield differential of 39.6 ± 7.5 l/150 d of milk (P<0.001) between the most (N2+Z7) and the least (N5+Z2) productive one (**Table IV-9**). The ewes with N2+Z7 genotype yielded 24.7 ± 6.9 l/150 d more milk (P<0.001) than ewes with N3+Z5 genotype which were found to be the most abundant in the studied population (9.0%). By flock, the major significant differences in milk yield of animals with different associated *GH2-N* and *GH2-Z* genotypes were as follows: N2+Z7 ewes from FL3 yielded respectively 84.2 ± 12.7 l/150 d and 78.2 ± 11.0 l/150 d more milk than N8+Z4 and N3+Z5 ewes (P<0.001); N18+Z20 ewes from FL5 yielded 48.6 ± 15.7 l/150 d more milk than N20+Z21 ewes (P<0.01); N4+Z9, N7+Z10 and N8+Z4 ewes from FL6 yielded significantly more milk (P<0.05) than N1+Z24, N2+Z25 and N5+Z2 ewes, with N8+Z4 ewes yielding 74.1 ± 17.2 l/150 d more milk than N2+Z25 ewes (P<0.001); N3+Z5 ewes from FL7 yielded respectively 32.6 ± 16.1 l/150 d, 34.8 ± 11.8 l/150 d and 38.4 ± 15.2 l/150 d more milk than N2+Z7, N5+Z2 and N6+Z2 ewes (P<0.05). No significant milk yield differences were found between the associated *GH2-N* and *GH2-Z* genotypes at FL1+FL4 and FL2 flocks. The ewes from F3 with the N2+Z7 genotype were found to be the best producing ones only in this flock; but not in flocks FL5 and FL7 where N2+Z7 ewes presented quite low milk yields. The N3+Z5 genotype seems to be a quite consensual high producing genotype, being the highest producing one from FL1+FL4 and FL7, and one of the highest from FL5; however it was one of the worst genotypes from FL3. Thus, the contradictory results shown above also appear to occur with the associated *GH2-N* and *GH2-Z* genotypes.

Table IV-9. *Associated* GH2-N+GH2-Z *genotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent* GH2-N+GH2-Z *genotype.*

GH2-N* + *GH2-Z	Freq. (%)	**All flocks** (N=818)	Freq. (%)	**FL1+FL4** (N=216)	Freq. (%)	**FL2** (N=159)	Freq. (%)	**FL3** (N=124)	Freq. (%)	**FL5** (N=190)	Freq. (%)	**FL6** (N=164)	Freq. (%)	**FL7** (N=88)
N1+Z1	3.5	6.5 ± 6.9 b			5.8	-12.4 ± 14.0			10.1	-14.0 ± 14.3 abc				
N1+Z6	2.0	9.9 ± 8.2 bc			5.3	-10.6 ± 16.8	5.2	46.6 ± 14.4 b						
N1+Z15									7.6	-17.4 ± 14.9 abc				
N1+Z18									4.6	-14.2 ± 16.5 abc				
N1+Z19									3.7	-13.9 ± 17.8 abc				
N1+Z24											5.0	8.3 ± 11.6 a		
N2+Z7	3.1	24.7 ± 6.9 c					9.0	78.2 ± 11.0 c	7.0	-22.2 ± 15.2 ab			4.5	-32.6 ± 16.1 a
N2+Z22									3.7	-3.8 ± 18.2 bc				
N2+Z25											4.3	-18.6 ± 14.3 a		
N3+Z5	9.0	0 b	4.1	0.0	16.4	0	26.1	0 a	3.1	0 bc			13.0	0 b
N4+Z9	6.3	-1.5 ± 6.0 b	3.6	-18.8 ± 12.2	11.6	9.8 ± 11.2					17.1	35.1 ± 8.4 b	5.1	-32.3 ± 17.6 ab
N5+Z2	6.6	-14.9 ± 5.8 a	3.6	-18.1 ± 11.8							20.7	0 a	13.0	-34.8 ± 11.8 a
N6+Z2	3.3	-2.1 ± 6.7 ab	6.4	-4.8 ± 10.7	3.4	0.0 ± 16.8							6.2	-38.4 ± 15.2 a
N6+Z8			4.6	-18.0 ± 11.8										
N7+Z10	5.1	11.3 ± 6.2 bc	6.2	-5.7 ± 11.8					7.0	-11.1 ± 15.2 bc	7.5	54.9 ± 10.4 b	7.9	-14.0 ± 14.5 ab
N8+Z4	3.0	-0.3 ± 6.8 ab					11.8	-6.0 ± 9.9 a	3.1	-14.3 ± 17.6 abc	3.9	55.5 ± 13.5 b		
N9+Z12	3.4	8.2 ± 6.9 bc	7.4	-7.0 ± 10.6	5.3	0.9 ± 14.0								
N10+Z14	1.9	12.5 ± 8.2 bc					6.6	49.3 ± 11.5 b						
N11+Z11	2.1	0.9 ± 8.3 ab			9.2	11.9 ± 11.2								
N12+Z2	1.9	11.9 ± 8.3 bc			11.1	1.3 ± 11.2								
N14+Z3			5.9	-7.3 ± 11.0										
N17+Z5					8.7	2.2 ± 11.2								
N18+Z20									4.3	8.9 ± 16.8 c				
N19+Z17			2.6	-21.5 ± 13.4										
N20+Z21									4.0	-39.7 ± 16.6 a				
N21+Z12			4.1	-11.8 ± 11.2										
N22+Z16			3.1	-3.5 ± 13.1										
N23+Z13			3.8	-12.7 ± 12.0										

Note: Genotypes with less than 2% of the total lactations are not shown. For *GH2-N* and *GH2-Z* genotype codes details see **Table IV-7** and **Table IV-8**.
N = number of lactations.
a, b, c – values in the same column with different letters are significantly different (P<0.05).

Seven GH2-N phenotypes were established in "Serra da Estrela" ewes (**Table IV-10**). The most frequent phenotypes were AA_N (70.0%) and AD_N (18.2%). More phenotypes do exist, but they could not be clearly established as they were found in animals carrying ambiguous haplotypes to which correspond two possible phenotypes; e.g. AH_N and/or BE_N (2.4%). Within GH2-N phenotypes there was a milk yield differential of 22.6 ± 6.8 l/150 d ($P<0.01$) to 31.4 ± 9.0 l/150 d ($P<0.001$) between the most producing one (AB_N) and all the others, i.e., AA_N, AC_N, AD_N, AF_N and AH_N and/or BE_N. By flock, the major significant differences in milk yield of animals with different GH2-N phenotypes were as follows: AF_N and AA_N ewes from FL3 yielded respectively 28.9 ± 10.7 l/150 d and 21.1 ± 5.7 l/150 d more milk than AD_N ewes ($P<0.001$); AB_N ewes from FL5 yielded respectively 48.1 ± 15.8 l/150 d and 44.8 ± 13.4 l/150 d more milk than AF_N ewes and AH_N and/or BE_N ewes ($P<0.05$); AB_N and AD_Newes from FL7 yielded respectively 49.4 ± 12.0 l/150 d ($P<0.001$) and 31.1 ± 8.3 l/150 d more milk than AA_N ewes ($P<0.001$). No significant milk yield differences were found between GH2-N phenotypes at FL1+FL4, FL2 and FL6 flocks. Conflicting results were observed again between FL3 flock and all the others: ewes from FL3 flock with AD_N phenotype produce low levels of milk; ewes from FL7 flock with the same phenotype produce medium levels and ewes from FL1+Fl4, FL2 and FL6 flocks carrying this phenotype were the best milk producers.

Ten GH2-Z phenotypes were established in "Serra da Estrela" ewes (**Table IV-11**). The more frequent phenotypes were AA_Z (45.5%), AE_Z (17.1%) and AB_Z (13.6%). More phenotypes exist, but they could not be clearly established as they were found in animals carrying ambiguous haplotypes to which correspond two possible phenotypes; e.g. AD_Z and/or BE_Z (3.4%). Within GH2-Z phenotypes there was a milk yield differential of 16.6 ± 8.3 l/150 d ($P<0.05$) between the most (BD_Z) and the least (AD_Z and/or BE_Z) producing one, with BD_Z having similar production levels to AA_Z and AE_Z phenotypes ($P>0.05$). By flock, the major significant differences in milk yield of animals with different GH2-Z phenotypes were as follows: AH_Z and/or CI_Z ewes from FL1+FL4 yielded 26.5 ± 12.6 l/150 d more milk than AD_Z and/or BE_Z ($P<0.05$); AI_Z ewes from FL3 yielded 53.5 ± 13.0 l/150 d more milk than AE_Z ewes ($P<0.001$); AE_Z ewes from FL5 yielded respectively 29.9 ± 10.1 l/150 d and 33.4 ± 12.9 l/150 d more milk than BB_Z and BD_Z ewes ($P<0.05$); BD_Z ewes from FL6 yielded 71.7 ± 16.8 l/150 d more milk than AD_Z and/or BE_Z ($P<0.001$); AB_Z and AE_Z ewes from FL7 yielded respectively 46.1 ± 13.3 l/150 d ($P<0.05$) and 54.2 ± 12.7 l/150 d ($P<0.01$) more milk than BB_Z ewes. No significant milk yield differences were found between *GH2-Z* phenotypes at FL2 flock. AE_Z, AI_Z and BD_Z were the best producing genotypes for milk in some flocks and the worst in other; again, results from FL3 flock largely contributed to these conflicting results.

Table IV-10. GH2-N *phenotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent homozygous* GH2-N *phenotype.*

GH2-N Phenotypes		Freq. (%)	**All flocks** (N=1634)	Freq. (%)	**FL1+FL4** (N=480)	Freq. (%)	**FL2** (N=212)	Freq. (%)	**FL3** (N=217)	Freq. (%)	**FL5** (N=340)	Freq. (%)	**FL6** (N=277)	Freq. (%)	**FL7** (N=161)
AA_N	**(a,a,a,a,a)/(a,a,a,a,a)**	70.0	0 a	78.66	0	68.0	0	39.6	0 b	75.9	0 b	77.5	0	67.8	0 a
AB_N	**(a,a,a,a,a)/(a,a,b,a,a)**	2.0	22.6 ± 6.8 b							5.3	21.5 ± 9.7 c			6.2	49.4 ± 12.0 b
AC_N	**(a,a,a,a,a)/(a,a,c,a,a)**	2.1	-6.8 ± 7.0 a	3.1	-8.5 ± 8.7							5.1	-20.4 ± 11.7		
AD_N	**(a,a,a,a,a)/(a,d,a,a,a)**	18.2	-1.2 ± 2.7 a	13.9	1.4 ± 4.6	25.0	2.3 ± 6.4	44.5	-21.1 ± 5.7 a	7.4	4.8 ± 8.5 bc	11.9	3.2 ± 7.1	16.9	31.1 ± 8.3 b
AF_N	**(a,a,a,a,a)/(a,b,a,a,a)**	2.4	-4.0 ± 6.4 a					6.2	7.8 ± 11.5 b	3.5	-26.6 ± 12.9 a				
AH_N or BE_N	**(a/a,a/c,a/b,a/a,a/a)**	2.4	-8.8 ± 6.4 a					5.3	-21.1 ± 14.4 ab	5.3	-23.2 ± 10.1 a				
AE_N	**(a,a,a,a,a)/(a,c,a,a,a)**									2.6	3.5 ± 13.3 abc				
AI_N	**(a,a,a,a,a)/(a,e,a,a,a)**			2.2	-1.5 ± 10.7										

Note: Phenotypes with less than 2% of the total lactations are not shown.

Phenotypes notation: phenotypes were defined as the product of the gene and denoted by capital letters corresponding to protein variants whenever they could be predicted, e.g. AA_N – five phase phenotype of the GH2-N copy composed of two protein variants V_NA (a,a,a,a,a) separated by /; individual fragments (II to VI) of protein variants were represented by a regular letter and were separated by a comma; it corresponds to a homozygous animal for V_NA. When an unique phenotype could not be predicted, unphased phenotypes were presented with individual fragment (II to VI) variants separated by a comma, e.g phenotype (a/a,a/c,a/b,a/a,a/a) where two combinations were possible: AH_N or BE_N. For individual fragments of protein variant details see **Table IV-5 and Table IV-6**.

N = number of lactations.

a, b, c – values in the same column with different letters are significantly different (P<0.05).

Table IV-11. GH2-Z *phenotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent homozygous* GH2-Z *phenotype.*

GH2-Z Phenotypes		Freq. (%)	**All flocks** (N=1554)	Freq. (%)	**FL1+FL4** (N=395)	Freq. (%)	**FL2** (N=206)	Freq. (%)	**FL3** (N=213)	Freq. (%)	**FL5** (N=322)	Freq. (%)	**FL6** (N=274)	Freq. (%)	**FL7** (N=165)
AA_Z	**(a,a,a,a,a)/(a,a,a,a,a)**	45.5	0 b	47.0	0 ab	54.8	0	22.5	0 b	34.8	0 ab	62.6	0 bc	52.0	0 b
AB_Z	**(a,a,a,a,a)/(a,b,a,a,a)**	13.6	-1.8 ± 3.0 ab	11.6	-6.6 ± 5.0 ab			9.0	-5.5 ± 10.1 b	29.8	-0.8 ± 5.3 ab	10.4	-6.5 ± 7.9 bc	15.3	19.0 ± 8.8 c
AC_Z	**(a,a,a,a,a)/(a,a,a,a,b)**	4.2	-1.4 ± 5.4 ab	17.3	0.5 ± 4.5 ab										
AE_Z	**(a,a,a,a,a)/(a,a,b,a,a)**	17.1	0.1 ± 2.9 b	7.4	4.5 ± 6.4 ab	30.8	-0.3 ± 6.3	45.0	-27.7 ± 6.9 a	7.7	16.1 ± 8.6 b	8.7	10.7 ± 9.1 cd	18.6	27.1 ± 8.1 c
AD_Z or BE_Z	**(a/a,a/b,a/b,a/a,a/a)**	3.4	-13.1 ± 5.4 a	3.5	-12.9 ± 8.9 a					5.4	-2.7 ± 10.7 ab	4.5	-35.7 ± 12.6 a		
AI_Z	**(a,a,a,a,a)/(a,c,a,a,a)**	2.3	-0.8 ± 6.4 ab			7.7	-18.4 ± 11	5.4	25.8 ± 12.8 c						
BB_Z	**(a,b,a,a,a)/(a,b,a,a,a)**	6.1	-0.2 ± 4.3 ab					14.9	13.3 ± 9.2 bc	13.4	-13.8 ± 7.3 a			7.3	-27.1 ± 11.4 a
BD_Z	**(a,b,a,a,a)/(a,b,b,a,a)**	2.0	3.6 ± 6.4 b							4.8	-17.3 ± 10.8 a	3.8	36.0 ± 11.7 d		
CC_Z	**(a,a,a,a,b)/(a,a,a,a,b)**			4.5	-2.2 ± 8.2 ab										
BG_Z	**(a,b,a,a,a)/(a,b,a,a,b)**			3.2	-7.2 ± 9.0 ab										
AH_Z or CI_Z	**(a/a,a/c,a/a,a/a,a/b)**			3.2	13.6 ± 9.3 b										
JJ_Z	**(a,a,c,a,a)/(a,a,c,a,a)**											4.8	-21.4 ± 11.2 ab		

Note: Phenotypes with less than 2% of the total lactations are not shown.

Phenotypes notation: phenotypes were defined as the product of the gene and denoted by capital letters corresponding to protein variants whenever they could be predicted, e.g. AA_Z – five phase phenotype of the GH2-Z copy composed of two protein variants V_ZA (a,a,a,a,a) separated by /; individual fragments (II to VI) of protein variants were represented by a regular letter and were separated with a comma; it corresponds to an homozygous animals for V_ZA. When an unique phenotype could not be predicted, unphased phenotypes were presented with individual fragment (II to VI) variants separated with a comma, e.g phenotype (a/a,a/c,a/a,a/a,a/b) where two combinations were possible: AH_Z or CI_Z. For individual fragments of protein variant details see **Table IV-5 and Table IV-6**.

N = number of lactations.

a, b, c, d – values in the same column with different letters are significantly different (P<0.05).

Table IV-12. *Associated* GH2-N+GH2-Z *phenotypes, their respective frequencies (%) and milk yield deviation (l/150 d) ± standard error from the most frequent homozygous* GH2-N+GH2-Z *phenotype.*

GH2-N* + *GH2-Z	Freq. (%)	**All flocks** (N=1369)	Freq. (%)	**FL1+FL4** (N=377)	Freq. (%)	**FL2** (N=194)	Freq. (%)	**FL3** (N=199)	Freq. (%)	**FL5** (N=292)	Freq. (%)	**FL6** (N=235)	Freq. (%)	**FL7** (N=138)
AA_N+AA_Z	42.1	0 ab	43.1	0 ab	54.3	0	17.1	0 a	33.9	0 b	56.7	0 b	47.5	0 ab
AA_N+AB_Z	10.0	-4.6 ± 3.5 ab	10.6	-8.1 ± 5.2 a					22.6	-3.6 ± 5.7 ab	10.4	-12.6 ± 8.0 ab	7.3	22.7 ± 12.5 bc
AA_N+AC_Z	2.4	2.2 ± 7.1 ab	9.6	3.9 ± 5.7 ab										
AA_N+AI_Z	2.3	1.3 ± 6.8 ab			7.7	-17.8 ± 11.1	5.4	49.6 ± 13.6 cd						
AA_N+BB_Z	3.3	7.9 ± 5.8 b					9.5	45.8 ± 11.0 cd	6.8	-9.5 ± 9.4 ab			6.2	-22.8 ± 12.4 a
AD_N+AA_Z	2.0	3.6 ± 6.8 ab	4.0	-4.0 ± 8.2 ab			5.4	62.6 ± 12.5 d			5.9	-26.1 ± 9.6 a		
AD_N+AC_Z			7. 7	-4.7 ± 6.3 ab										
AD_N+AE_Z	14.2	0.1 ± 3.4 ab	5.0	11.2 ± 7.9 b	25.8	0.4 ± 6.8	41.4	-15.1 ± 7.8 a	6.0	5.2 ± 9.5 bc	5.2	23.9 ± 10.9 c	16.9	32.5 ± 8.7 c
AF_N+AB_Z	2.1	0.7 ± 7.2 ab					6.3	26.7 ± 12.0 bc						
(AH_N or BE_N)+BB_Z	2.5	-8.3 ± 6.6 a					5.4	-1.7 ± 14.2 ab	5.4	-24.9 ± 10.4 a				
AA_N+(AD_Z or BE_Z)	2.1	-5.7 ± 7.1 ab							4.5	7.9 ± 11.6 bc				
AA_N+CC_Z			4.5	-2.5 ± 8.3 ab										
AA_N+BG_Z			3.2	-7.9 ± 9.1 ab										
AA_N+BD_Z									3.3	-20.8 ± 13.4 ab				
AA_N+(AH_Z or CI_Z)			3.2	13.1 ± 9.4 b										
AB_N+AB_Z									4.5	22.1 ± 10.9 c				
AC_N+(AD_Z or BE_Z)			2.5	-11.8 ± 10.2 ab										
AD_N+JJ_Z											3.1	8.8 ± 13.9 bc		

Note: Phenotypes with less than 2% of the total lactations are not shown. For GH2-N and GH2-Z phenotypes codes details see **Table IV-10** and **Table IV-11**.
N = number of lactations.
a, b, c, d – values in the same column with different letters are significantly different (P<0.05).

The effect of associated GH2-N and GH2-Z phenotypes revealed a milk yield differential of 16.2 ± 8.1 l/150 d of milk (P<0.05) between the most (AA_N+BB_Z) and the least [(AH_N and/or BE_N)+BB_Z] producing phenotype (**Table IV-12**). The ewes with AA_N+BB_Z phenotype yielded 7.9 ± 5.8 l/150 d more milk (P>0.05) than ewes with AA_N+AA_Z (42.1%) and AD_N+AE_Z (14.19%) phenotypes which were the two most abundant in the studied population and presented similar milk yields between them (P>0.05). By flock, the major significant differences in milk yield were as follows: AD_N+AE_Z and [AA_N+(AH_Z and/or CI_Z)] ewes from FL1+FL4 yielded respectively 19.3 ± 8.9 l/150 d and 21.2 ± 10.3 l/150 d more milk than AA_N+AB_Z ewes (P<0.05); AD_N+AA_Z ewes from FL3 yielded respectively 62.6 ± 12.5 l/150 d and 77.7 ± 11.6 l/150 d more milk than AD_N+AE_Z ewes (P<0.001); AB_N+AB_Z ewes from FL5 yielded 47.0 ± 14.0 l/150 d more milk than [(AH_N and/or BE_N)+BB_Z] ewes (P<0.01); AD_N+AE_Z ewes from FL6 yielded 50.0 ± 13.5 l/150 d more milk than AD_N+AA_Z ewes (P<0.01); AD_N+AE_Z ewes from FL7 yielded 56.3 ± 13.9 l/150 d more milk than AA_N+BB_Z ewes (P<0.01). No significant milk yield differences were found between the associated GH2-N and GH2-Z phenotypes at FL2 flock. Ewes with AD_N+AE_Z phenotype were the best producing ones in FL6 and FL7 flocks, one of the best in FL1+FL4 and the worst in FL3.

IV.2.5.2 Milk composition

The records of milk fat and protein contents and yields were available for 294 ewes for one year (1998) and their mean milk production adjusted to 150 lactation days (± SD) on that year was 171.4 ± 75.9 l with 79.5 ± 11.8 g of fat/kg of milk and 65.5 ± 7.2 g of protein/kg of milk. Mean fat yield was 13.8 ± 6.7 kg, mean protein yield was 11.1 ± 4.7 kg and mean fat+protein yield was 25.0 ± 11.3 kg. The statistical analyses were performed considering the *GH2-N* and *GH2-Z* copies genotypes (**Table IV-13**) and phenotypes (**Table IV-15**) taken separately or associated (**Table IV-14** and **Table IV-15**) in the population. Contrasts were performed considering the differences between the different genotypes or phenotypes for each parameter and the most frequent homozygous genotype or phenotypes in each o*GH* gene copy, or associated *GH2-N* and *GH2-Z* genotypes or phenotypes. Genotypes and phenotypes with frequencies lower than 2% were excluded from the analysis. The genotypes and phenotypes found in the *GH2-N* and *GH2-Z* copies had significant influence on milk yield traits and on milk fat and protein contents in "Serra da Estrela" ewes (P<0.05). Exceptions were found for *GH2-N* genotype effects on protein content and for GH2-N phenotype effects on milk yield, fat and protein content, and fat+protein yield and for GH2-Z phenotype effects on fat content (P>0.05). The major significant effects are described below.

Table IV-13. GH2-N *and* GH2-Z *genotypes, their respective frequencies (%) and deviation of milk yield (l/150 d), fat and protein contents (g/kg), and fat, protein and fat+protein yields (kg/150 d) at 150 days of lactation ± standard error for the most frequent homozygous GH2-N and GH2-Z genotype.*

***GH2-N* genotype (N=193)**		**Freq. (%)**	**Milk yield (l/150 d)**	**Fat content (g/kg)**	**Protein content (g/kg)**	**Fat yield (kg/150 d)**	**Protein yield (kg/150 d)**	**Fat+Protein yield (kg/150 d)**
N1	*(1,1,1,1,1,1,1)/(1,1,1,2,1,1,1)*	7.8	-20.3 ± 9.5 a	-3.89 ± 3.25 abc	-0.68 ± 3.17	-2.92 ± 0.66 a	-1.78 ± 0.60 ab	-4.69 ± 1.33 a
N2	*(1,1,1,1,1,1,1)/(1,1,1,1,1,1,1)*	5.8	0 bc	0 b	0	0 c	0 d	0 c
N3	*(1/1,1/1,1/5,1/1,1/1,1/1,2/4)*	9.5	-7.8 ± 9.1 abc	-3.51 ± 3.11 abc	-2.20 ± 3.04	-1.99 ± 0.63 abc	-1.29 ± 0.57 abc	-3.31 ± 1.27 ab
N4	*(1/1,1/1,1/4,1/2,1/1,1/1,1/1)*	8.2	-10.6 ± 9.6 abc	-4.15 ± 3.30 abc	-3.25 ± 3.21	-2.07 ± 0.67 abc	-1.28 ± 0.61 abc	-3.39 ± 1.35 ab
N5	*(1/1,1/1,1/1,1/2,1/1,1/1,2/4)*	7.5	-17.0 ± 9.7 ab	-3.22 ± 3.33 abc	-2.81 ± 3.24	-2.26 ± 0.68 abc	-1.61 ± 0.61 abc	-3.90 ± 1.36 ab
N6	*(1/1,1/1,1/1,1/2,1/1,2/3,1/5)*	5.4	3.3 ± 11.1 bc	-7.98 ± 3.82 a	-4.46 ± 3.72	-1.33 ± 0.77 bcd	-0.68 ± 0.70 bcd	-2.08 ± 1.55 abc
N7	*(1/1,1/1,1/4,1/2,1/1,1/1,6/7)*	4.4	5.4 ± 11.6 c	-6.79 ± 4.00 ab	-2.90 ± 3.90	-1.29 ± 0.81 bcd	-0.27 ± 0.73 cd	-1.62 ± 1.62 bc
N8	*(1,1,1,1,1,1,1)/(1,1,5,1,1,1,1)*	2.7	-18.2 ± 13.4 abc	3.16 ± 4.61 c	-4.46 ± 4.49	-1.41 ± 0.93 abcd	-2.08 ± 0.84 ab	-3.40 ± 1.88 abc
N9	*(1/1,1/1,1/4,1/2,1/1,2/3,3/3)*	4.4	3.9 ± 11.4 c	-4.15 ± 3.91 abc	-3.89 ± 3.81	-0.88 ± 0.79 cd	-0.63 ± 0.71 bcd	-1.55 ± 1.59 bc
N11	*(1/1,1/1,1/4,1/2,1/1,2/3,1/5)*	2.0	0.1 ± 14.5 abc	-1.12 ± 5.02 abc	-3.71 ± 4.90	-0.93 ± 1.01 bcd	-0.85 ± 0.91 abcd	-1.85 ± 2.03 abc
N12	*(2/3,1/1,1/1,1/2,1/1,1/1,2/4)*	3.1	9.1 ± 12.9 c	-2.59 ± 4.47 abc	-8.44 ± 4.36	0.19 ± 0.90 c	-0.97 ± 0.81 abcd	-0.83 ± 1.80 bc
N14	*(1/1,1/2,1/1,1/2,1/1,4/5,2/2)*	2.4	-1.6 ± 14.5 abc	-8.07 ± 4.96 abc	-4.36 ± 4.84	-1.45 ± 1.01 abcd	-0.76 ± 0.91 abcd	-2.24 ± 2.02 abc
N18	*(1,1,1,1,1,1,1)/(1,1,1,3,1,1,1)*	2.4	-17.4 ± 13.5 abc	-7.59 ± 4.61 abc	-6.91 ± 4.49	-3.20 ± 0.94 ab	-2.46 ± 0.85 a	-5.73 ± 1.89 a
***GH2-Z* genotype (N=202)**								
Z1	*(1/1,1/1,1/1,1/1,1/2,2/3)*	4.1	-7.3 ± 13.2 b	3.18 ± 4.53 ab	1.99 ± 4.42 ab	-0.56 ± 0.92 bc	-0.38 ± 0.83 abc	-0.90 ± 1.84 b
Z2	*(1/1,1/1,1/2,1/1,1/2,2/3)*	14.6	-0.4 ± 10.8 b	0.88 ± 3.72 a	-0.32 ± 3.62 a	-0.03 ± 0.75 bc	-0.2 ± 0.68 abc	-0.24 ± 1.51 b
Z3	*(1,3,1,1,1,1)/(1,3,1,1,1,1)*	3.4	0 b	0 a	0 ab	0 bc	0 abc	0 b
Z4	*(1,1,1,1,1,2)/(1,1,1,1,2,2)*	4.1	8.5 ± 14.1 b	4.81 ± 4.85 ab	-0.54 ± 4.73 ab	0.97 ± 0.98 c	0.21 ± 0.89 abc	1.23 ± 1.97 bc
Z5	*(1,3,1,1,1,3)/(1,3,1,1,1,3)*	12.2	-1.5 ± 11.7 b	1.56 ± 4.05 ab	1.96 ± 3.95 ab	-0.48 ± 0.82 b	-0.24 ± 0.74 abc	-0.72 ± 1.64 b
Z6	*(1,3,1,1,1,4)/(1,3,1,1,2,4)*	2.7	-37.6 ± 15.0 a	0.14 ± 5.15 a	8.67 ± 5.03 b	-3.59 ± 1.04 a	-1.51 ± 0.94 a	-5.09 ± 2.09 a
Z7	*(1/1,1/1,1/2,1/1,1/2,2/2)*	2.4	16.6 ± 15.9 b	11.37 ± 5.49 b	2.19 ± 5.35 ab	3.60 ± 1.11 d	1.31 ± 1 b	4.93 ± 2.23 c
Z8	*(1/1,2/2,1/3,1/1,1/2,2/2)*	2.4	-4.1 ± 14.6 b	0.31 ± 4.99 ab	-2.62 ± 4.87 a	-0.51 ± 1.01 bc	-0.71 ± 0.92 ab	-1.22 ± 2.03 ab
Z9	*(1/1,3/3,1/1,1/1,1/2,2/3)*	6.8	-9.6 ± 12.4 b	1.59 ± 4.24 a	1.31 ± 4.13 ab	-0.97 ± 0.86 b	-0.57 ± 0.78 ab	-1.53 ± 1.73 b
Z10	*(1/1,1/5,1/1,1/1,1/2,2/2)*	4.1	10.0 ± 13.1 b	-2.23 ± 4.48 a	0.72 ± 4.37 ab	0.05 ± 0.91 bc	0.61 ± 0.82 bc	0.62 ± 1.82 b
Z11	*(1,1,1,1,1,4)/(1,1,1,1,3,4)*	2.7	-5.0 ± 14.6 b	6.53 ± 5.03 ab	2.29 ± 4.9 ab	0.27 ± 1.02 bc	-0.11 ± 0.92 abc	0.13 ± 2.04 b
Z12	*(1/1,2/3,1/1,1/1,1/2,2/3)*	6.5	0.2 ± 11.5 b	4.04 ± 3.94 ab	1.89 ± 3.84 ab	0.17 ± 0.80 bc	0.02 ± 0.72 abc	0.20 ± 1.61 b
Z13	*(1/1,1/1,1/1,1/1,1/3,2/3)*	2.7	0.3 ± 13.6 b	-0.56 ± 4.67 a	0.42 ± 4.56 ab	-0.26 ± 0.95 bc	-0.06 ± 0.85 abc	-0.31 ± 1.90 b

Note: Genotypes with less than 2% of the total lactations are not shown. For details of *GH2-N* and *GH2-Z* genotype codes see **Table IV-4**.

N = number of lactations.

a, b, c, d – values in the same column with different letters are significantly different (P<0.05).

Table IV-14. *Associated* GH2-N+GH2-Z *genotypes, their respective frequencies (%) and deviation of milk yield (l/150 d), fat and protein contents (g/kg), and fat, protein and fat+protein yields (kg/150 d) at 150 days of lactation ± standard error for the most frequent homozygous associated GH2-N+GH2-Z genotype.*

***GH2-N+GH2-Z* genotypes (N=150)**	**Freq. (%)**	**Milk yield (l/150 d)**	**Fat content (g/kg)**	**Protein content (g/kg)**	**Fat yield (kg/150 d)**	**Protein yield (kg/150 d)**	**Fat+Protein yield (kg/150 d)**
N3+Z5	9.5	0 cd	0 a	0 ab	0 bc	0 ab	0 bc
N5+Z2	7.5	-19.7 ± 9.6 ab	0.29 ± 3.30 ab	0.52 ± 3.21 ab	-1.05 ± 0.67 b	-0.77 ± 0.60 a	-1.82 ± 1.34 ab
N4+Z9	6.8	-11.3 ± 9.7 abc	0.15 ± 3.35 ab	0.47 ± 3.26 ab	-0.57 ± 0.67 bc	-0.31 ± 0.61 ab	-0.88 ± 1.35 abc
N9+Z12	4.4	2.0 ± 10.6 cd	-0.33 ± 3.67 abc	-0.50 ± 3.58 ab	0.43 ± 0.74 cd	0.25 ± 0.67 ab	0.68 ± 1.48 bc
N7+Z10	4.1	7.4 ± 11.0 cd	-3.66 ± 3.80 a	0.13 ± 3.71 ab	0.28 ± 0.77 bcd	0.83 ± 0.69 b	1.09 ± 1.54 bc
N6+Z2	2.7	8.8 ± 12.7 cde	-4.14 ± 4.39 a	-0.94 ± 4.28 ab	0.60 ± 0.88 bcd	0.74 ± 0.80 ab	1.32 ± 1.77 bc
N8+Z4	2.7	-11.8 ± 12.6 abcd	9.55 ± 4.36 bc	-1.95 ± 4.25 ab	1.11 ± 0.88 cd	-0.74 ± 0.79 ab	0.50 ± 1.76 bc
N12+Z2	2.7	18.0 ± 12.3 de	0.26 ± 4.27 abc	-6.54 ± 4.16 a	2.17 ± 0.86 d	0.31 ± 0.77 ab	2.47 ± 1.72 c
N14+Z3	2.4	-0.1 ± 14.4 abcd	-3.77 ± 4.93 a	-1.22 ± 4.81 ab	0.20 ± 1.00 bcd	0.33 ± 0.90 ab	0.53 ± 2.01 abc
N2+Z7	2.0	40.1 ± 13.3 de	10.47 ± 4.59 c	0.98 ± 4.48 ab	6.13 ± 0.93 e	3.21 ± 0.84 c	9.36 ± 1.86 d
N1+Z6	2.0	-36.0 ± 13.3 a	-2.11 ± 4.60 a	6.73 ± 4.49 b	-3.14 ± 0.93 a	-1.18 ± 0.84 a	-4.26 ± 1.86 a
N6+Z8	2.0	-2.6 ± 14.5 abcd	-2.31 ± 4.99 abc	-3.10 ± 4.87 ab	-0.15 ± 1.01 bcd	-0.28 ± 0.91 ab	-0.44 ± 2.03 abc
N11+Z11	2.0	1.4 ± 14.5 bcd	2.44 ± 5.03 abc	0.26 ± 4.90 ab	0.56 ± 1.01 bcd	0.28 ± 0.91 ab	0.81 ± 2.02 bc

Note: Genotypes with less than 2% of the total lactations are not shown. For details of *GH2-N* and *GH2-Z* genotype codes see **Table IV-13**.
N = number of lactations.
a, b, c, d, e – values in the same column with different letters are significantly different (P<0.05).

Table IV-15. *GH2-N, GH2-Z and associated GH2-N+GH2-Z phenotypes, their respective frequencies (%) and deviation of milk yield (l/150 d), fat and protein contents (g/kg), and fat, protein and fat+protein yields (kg/150 d) at 150 days of lactation ± standard error for the most frequent homozygous phenotype.*

	Freq. (%)	Milk yield (L/150 d)	Fat content (g/kg)	Protein content (g/kg)	Fat yield (kg/150 d)	Protein yield (kg/150 d)	Fat+Protein yield (kg/150 d)
GH2-N phenotype (N=284)							
AA_N	69.0	0	0	0	0 c	0 ab	0
AC_N	3.1	-11.8 ± 10.2	-1.77 ± 3.47	-2.38 ± 3.39	-1.35 ± 0.71 a	-1.08 ± 0.64 a	-2.47 ± 1.42
AD_N	20.4	0.0 ± 4.5	0.55 ± 1.54	-0.26 ± 1.50	-0.19 ± 0.31 b	-0.15 ± 0.28 ab	-0.32 ± 0.63
AF_N	2.0	9.7 ± 12.0	-3.44 ± 4.10	0.90 ± 4.00	-0.16 ± 0.83 bc	1.05 ± 0.75 b	0.97 ± 1.67
AI_N	2.0	-4 ± 11.9	0.14 ± 4.07	-0.44 ± 3.97	-0.34 ± 0.83 bc	0.03 ± 0.75 ab	-0.31 ± 1.66
GH2-Z phenotype (N=265)							
AB_Z	8.2	3.5 ± 6.5 b	-1.51 ± 2.22	-0.07 ± 2.16 a	-0.20 ± 0.45 b	0.13 ± 0.41 ab	-0.04 ± 0.90 bc
AD_Z or BE_Z	3.4	-17.3 ± 9.7 ab	-1.55 ± 3.33	0.29 ± 3.24 a	-1.35 ± 0.68 b	-1.02 ± 0.61 ab	-2.34 ± 1.36 ab
AA_Z	45.9	0 b	0	0 a	0 b	0 ab	0 b
AC_Z	6.5	3.6 ± 7.7 b	-1.25 ± 2.65	0.15 ± 2.59 a	0.21 ± 0.54 b	0.31 ± 0.49 ab	0.54 ± 1.08 bc
AE_Z	19.4	0.0 ± 4.9 b	0.22 ± 1.70	1.08 ± 1.66 a	-0.12 ± 0.34 b	0.00 ± 0.31 ab	-0.12 ± 0.69 b
BB_Z	4.1	1.7 ± 9.0 b	5.42 ± 3.08	1.08 ± 3.00 a	1.95 ± 0.63 c	0.57 ± 0.57 b	2.55 ± 1.26 c
AI_Z	2.7	-35.4 ± 10.6 a	-1.87 ± 3.66	7.51 ± 3.57 b	-3.33 ± 0.74 a	-1.35 ± 0.67 a	-4.68 ± 1.48 a
GH2-NZ phenotype (N=235)							
AA_N+AB_Z	6.1	-2.0 ± 7.4 b	-2.03 ± 2.52 a	-0.80 ± 2.46 a	-0.73 ± 0.51 b	-0.42 ± 0.46 ab	-1.14 ± 1.03 ab
AA_N+AA_Z	42.2	0 b	0 a	0 a	0 b	0 ab	0 b
AA_N+AC_Z	3.7	5.0 ± 9.8 b	-2.69 ± 3.35 a	0.57 ± 3.27 ab	0.25 ± 0.68 b	0.49 ± 0.61 ab	0.74 ± 1.36 bc
AA_N+AE_Z	2.0	5.9 ± 12.1 b	-4.89 ± 4.14 a	1.54 ± 4.04 ab	-0.03 ± 0.84 b	0.67 ± 0.76 ab	0.67 ± 1.70 bc
AA_N+BB_Z	2.7	8.5 ± 11.2 b	9.25 ± 3.84 b	1.67 ± 3.75 ab	2.83 ± 0.78 c	0.95 ± 0.70 b	3.81 ± 1.56 c
AA_N+AI_Z	2.7	-34.4 ± 10.7 a	-1.44 ± 3.69 a	7.80 ± 3.60 b	-3.24 ± 0.75 a	-1.24 ± 0.67 a	-4.46 ± 1.50 a
AD_N+AA_Z	2.0	-9.3 ± 12.1 ab	-1.07 ± 4.12 a	1.81 ± 4.02 ab	-1.23 ± 0.85 ab	-0.26 ± 0.77 ab	-1.41 ± 1.70 ab
AD_N+AC_Z	2.7	-1.7 ± 11.0 b	-0.98 ± 3.77 a	-1.38 ± 3.67 ab	-0.31 ± 0.76 b	-0.27 ± 0.69 ab	-0.56 ± 1.53 abc
AD_N+AE_Z	15.6	2.3 ± 5.6 b	1.33 ± 1.95 a	0.65 ± 1.90 ab	0.10 ± 0.39 b	0.04 ± 0.35 ab	0.17 ± 0.78 b

Note: Phenotypes with less than 2% of the total lactations are not shown. For details of GH2-N and GH2-Z phenotypes codes see **Table IV-5 and Table IV-6**.
N = number of lactations.
$^{a, b, c}$ – values in the same column with different letters are significantly different (P<0.05).

The analysis of *GH2-N* genotypes revealed that N7, N9 and N12 ewes yielded respectively 25.7 ± 10.5 l/150 d, 24.1 ± 10.2 l/150 d and 29.4 ± 11.9 l/150 d more milk than N1 ewes (P<0.05; **Table IV-13**). It was also found that the milk of the N8 ewes had significantly higher fat content 11.14 ± 4.93 g/kg than the milk of N6 ewes(P<0.05). N2 and N12 ewes yielded respectively 2.92 ± 0.66 kg/150 d and 3.39 ± 0.83 kg/150 d more fat than N1 ewes (P<0.01). N2 ewes yielded also 2.46 ± 0.85 kg/150 d (P<0.05) more protein and 5.73 ± 1.89 kg/150 d (P<0.01) more fat+protein than N18 ewes.

The analysis of *GH2-Z* genotypes revealed that Z7 ewes yielded 54.2 ± 15.6 l/150 d more milk than Z6 ewes (P<0.01), but not than the other ewes. The milk of the Z7 ewes had significantly higher fat content (P<0.05) than Z2, Z3, Z6, Z9, Z10 (more 13.60 ± 5.12 g/kg) and Z13 ewes, which milks have similar fat contents (P>0.05). The milk of the N6 ewes had respectively 8.99 ± 3.86 g/kg and 11.29 ± 5.12 g/kg higher protein content than the milk of Z2 and Z8 ewes (P<0.05). Z7 ewes yielded also significantly more fat (7.19 ± 1.08 kg/150 d; P<0.001), protein (2.82 ± 0.98 kg/150 d; P<0.05) and fat+protein (10.02 ± 2.18 kg/150 d; P<0.001) than Z6 ewes (**Table IV-13**).

The analysis of associated *GH2-N*+*GH2-Z* genotypes revealed that N2+Z7 ewes yielded significantly (P<0.001) more milk (76.1 ± 17.4 l/150 d), fat (9.27 ± 1.21 kg/150 d), protein (4.39 ± 1.10 kg/150 d) and fat+protein (13.62 ± 2.44 kg/150 d), and their milk had significantly higher fat content (12.57 ± 6.00 g/kg; P<0.05) than N1+Z6 ewes and that N2+Z7 and N1+Z6 ewes had identical protein content (**Table IV-14**). Furthermore, the milk of N2+Z7 ewes had also higher levels of fat content than the milk of N3+Z5, N7+Z10, N6+Z2 and N14+Z3 ewes. It was also observed that the milk of N1+Z6 ewes had significantly higher protein content (13.27 ± 5.44 kg/150 d; P<0.05) than the milk of N12+Z2 ewes.

The analysis of GH2-N phenotypes revealed that AF_N ewes yielded more milk (21.4 ± 15.6 l/150 d; P>0.05), fat (1.19 ± 1.09 kg/150 d; P<0.05), protein (2.13 ± 0.98 kg/150 d; P<0.05) and fat+protein (3.44 ± 2.18 kg/150 d; P>0.05) than AC_N ewes, and that these phenotypes exhibited identical milk fat and protein contents (**Table IV-15**). It was also observed that AA_N ewes yielded 1.35 ± 0.71 kg/150 d more fat than AC_N ewes (P<0.05).

The analysis of GH2-Z phenotypes revealed that AA_Z, AB_Z, AC_Z, AE_Z and BB_Z ewes yielded on average 37.2 ± 12.08 l/150 d more milk (P<0.05) than AI_Z ewes (**Table IV-15**). All phenotypes presented identical milk fat contents. The milk of AI_Z ewes had on average 7.09 ± 4.15 g/kg more protein contents than all the other ewes (P<0.05). The major

differences in yield traits were observed between BB_Z and AI_Z ewes, i.e., BB_Z ewes yielded more fat (5.28 ± 0.94 kg/150 d; P<0.001), more protein (1.92 ± 0.84 kg/150 d; P<0.05) and more fat+protein (7.23 ± 1.88 kg/150 d; P<0.05) than AI_Z ewes. This reflects the fact that BB_Z ewes yield more milk (more 37.1 ± 13.4 l/150 d than AI_Z ewes; P<0.05).

The analysis of the associated GH2-N+GH2-Z phenotypes revealed that AA_N+BB_Z ewes yielded more milk (42.9 ± 14.9 l/150 d; P<0.05), with higher fat content (10.69 ± 5.13 kg/150 d; P<0.05) than AA_N+AI_Z ewes and that these phenotypes have identical protein content; thus AA_N+BB_Z yielded significantly more fat (6.07 ± 1.04 kg/150 d; P<0.001), protein (2.19 ± 0.94 kg/150 d; P<0.05) and fat+protein (8.27 ± 2.08 kg/150 d; P<0.01) than AA_N+AI_Z ewes (**Table IV-15**).

Generally speaking, genotypes and phenotypes had a huge influence on milk yield traits, and only a moderate one on milk fat and protein contents. This is an interesting result as it can be expected to improve milk yield potential of the ewes without major detrimental impact on their milk quality. Moreover, it was observed simultaneously a higher level of milk yield and milk fat content in ewes with some genotypes/phenotypes (e.g. AA_N+BB_Z ewes).

IV.3 Single nucleotide polymorphisms (SNPs)

Sequencing of the *GH2-N* and *GH2-Z* copies disclosed SNPs (e.g. **Figure IV-8**) that could not be genotyped by PCR-SSCP analysis within the exon 5 of the *GH2-Z* gene copy ($E5Z_{1851}$).

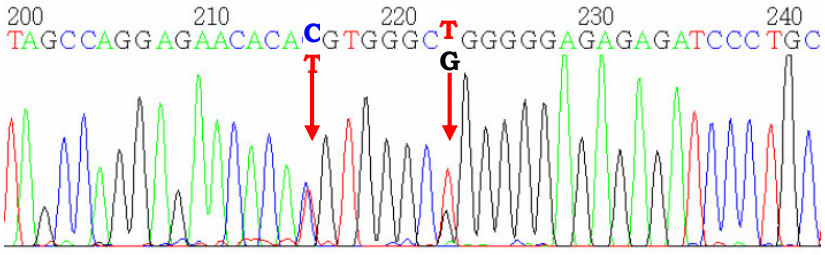

Figure IV-8. *Partial electropherogram of the PCR product of the* GH2-Z *copy presenting SNPs* $I4Z_{1551}$ *(C/T) and* $I4Z_{1558}$ *(T/G).*
The red arrows indicate the two polymorphic sites.

SNPs were also found in the 5'-UTR region and in intronic regions. Those SNPs and their genotype and allele frequencies for the sequenced animals are listed in **Table IV-16** and **Table IV-17**. More than 100 other polymorphic sites were found across the o*GH* gene copies, but only in one strand. Thus SNPs for those sites could not be validated and were not listed.

Hardy-Weinberg equilibrium was verified for almost all SNP loci within the *GH2-N* copy. Deviations towards heterozygote deficit were found for 5'$N_{(-125)}$ (P<0.01), for I2N_{793} (P<0.05) and for I3N_{1259} (P<0.05).

Table IV-16. GH1 *or* GH2-N *SNPs and their respective genotype and allele frequencies (%).*

Region	Site	Genotypes[a]						Alleles[b]			
5'	5'$N_{(-125)}$	AA	3 (0.15)	AG	2 (0.10)	GG	15 (0.75)	A	8 (0.20)	G	32 (0.80)
	5'$N_{(-61)}$	CC	0	CT	2 (0.10)	TT	18 (0.90)	C	2 (0.05)	T	38 (0.95)
I1	I1N_{381}	CC	16 (0.76)	CT	4 (0.19)	TT	1 (0.05)	C	36 (0.86)	T	6 (014)
	I1N_{427}	CC	18 (0.90)	CT	2 (0.10)	TT	0	C	38 (0.95)	T	2 (0.05)
	I1N_{431}	CC	0	CT	2 (0.10)	TT	18 (0.90)	C	2 (0.05)	T	38 (0.95)
	I1N_{437}	CC	0	CT	2 (0.10)	TT	18 (0.90)	C	2 (0.05)	T	38 (0.95)
	I1N_{452}	AA	0	AG	2 (0.10)	GG	18 (0.90)	A	2 (0.05)	G	38 (0.95)
	I1N_{458}	AA		A-		--		A	6	-	14
	I1N_{480}	AA	0	AG	5 (0.25)	GG	15 (0.15)	A	5 (0.12)	G	35 (0.88)
I2	I2N_{790}	AA		A-		--		A	17	-	3
	I2N_{793}	CC	14 (0.70)	CT	3 (0.15)	TT	3 (0.15)	C	31 (0.78)	T	9 (0.22)
	I2N_{811}	CC	1 (0.05)	CT	5 (0.25)	TT	14 (0.70)	C	7 (0.18)	T	33 (0.82)
	I2N_{854}	AA	2 (0.10)	AG	4 (0.20)	GG	14 (0.70)	A	8 (0.20)	G	32 (0.80)
	I2N_{926}	AA	1 (0.05)	AC	5 (0.25)	CC	14 (0.70)	A	7 (0.18)	C	33 (0.82)
I3	I3N_{1254}	CC	14 (0.70)	CT	5 (0.25)	TT	1 (0.05)	C	33 (0.82)	T	7 (0.18)
	I3N_{1259}	AA	8 (0.40)	AC	5 (0.25)	CC	7 (0.35)	A	21 (0.52)	C	19 (0.48)
	I3N_{1283}	AA	13 (0.65)	AG	6 (0.30)	GG	1 (0.05)	A	32 (0.80)	G	8 (0.20)
	I3N_{1289}	CC	1 (0.05)	CG	6 (0.30)	GG	13 (0.65)	C	8 (0.20)	G	32 (0.80)
I4	I4N_{1486}	AA	11 (0.55)	AG	7 (0.35)	GG	2 (0.10)	A	29 (0.72)	G	11 (0.28)
	I4N_{1509}	AA	11 (0.55)	AG	7 (0.35)	GG	2 (0.10)	A	29 (0.72)	G	11 (0.28)
	I4N_{1558}	GG	0	GT	6 (0.30)	TT	14 (0.14)	G	6 (0.15)	T	34 (0.85)
	I4N_{1648}	CC	17 (0.85)	CT	2 (0.10)	TT	1 (0.05)	C	36 (0.90)	T	4 (0.10)
	I4N_{1711}	AA	18 (0.90)	AG	2 (0.10)	GG	0	A	38 (0.95)	G	2 (0.05)

[a] number of animals and genotype frequencies (%) in brackets.
[b] number of alleles and allele frequencies (%) in brackets.
Site: U – UTR; E – exon; I – intron; *N* – *GH2-N* (or *GH1*) copy; e.g., 5'$N_{(-125)}$ – 5'-region at *GH2-N* (or *GH1*) copy, position 125 upstream from the transcription starting site; 5UN_{381} – 5'-UTR at *GH2-N* (or *GH1*) copy, position 381 according to the published *o*GH gene sequence (Orian *et al*., 1988); GenBank accession no. X12546.

HWE was also verified for some SNP loci within the *GH2-Z* copy. Deviations towards heterozygote deficit were found for 5'$Z_{(-326)}$ (P<0.001), for 5'$Z_{(-282)}$ (P<0.05), for 5'$Z_{(-227)}$ (P<0.05), for I1Z_{438} (P<0.05), for I2Z_{794} (P<0.05), for I2Z_{797} (P<0.05), and this tendency was also observed for I2N_{929} (P<0.10).

Polymorphic sites detected with PCR-SSCP analysis were also analysed individually for HWE. Deviations were only observed for E2Z_{712} and E5Z_{1852} (significant for heterozygote deficit; P<0.05).

Table IV-17. GH2-Z *SNPs and their respective genotype and allele frequencies (%).*

Region	Site	Gh1/Gh2 Genotypes[a]				Gh2/Gh2 Genotypes[a]						Alleles[b]			
5'	$5'Z_{(-326)}$	A-	1 (0.50)	G-	1 (0.50)	AA	4 (0.27)	AG	2 (0.13)	GG	9 (0.60)	A	11 (0.34)	G	21 (0.66)
	$5'Z_{(-304)}$			G-	2 (1.00)	AA	1 (0.06)	AG	3 (0.19)	GG	12 (0.75)	A	5 (0.15)	G	29 (0.85)
	$5'Z_{(-282)}$			G-	2 (1.00)	GG	9 (0.60)	GT	3 (0.20)	TT	3 (0.20)	G	21 (0.66)	T	11 (0.34)
	$5'Z_{(-267)}$	C-	2 (1.00)			CC	11 (0.73)	CT	3 (0.20)	TT	1 (0.07)	C	27 (0.84)	T	5 (0.16)
	$5'Z_{(-227)}$	A-	1 (0.50)	T-	1 (0.50)	AA	8 (0.53)	AT	4 (0.27)	TT	3 (0.20)	A	21 (0.66)	T	11 (0.34)
	$5'Z_{(-191)}$	C-	1 (0.50)	T-	1 (0.50)	CC	1 (0.07)	CT	5 (0.33)	TT	9 (0.60)	C	8 (0.25)	T	24 (0.75)
	$5'Z_{(-107)}$	C-	1 (0.50)	G-	1 (0.50)	CC	1 (0.07)	CG	5 (0.33)	GG	9 (0.60)	C	8 (0.25)	G	24 (0.75)
5'-UTR	$5UZ_{288}$			T-	2 (1.00)	CC	2 (0.13)	CT	4 (0.27)	TT	9 (0.60)	C	8 (0.25)	T	24 (0.75)
I1	$I1Z_{438}$	A-	1 (0.50)	G-	1 (0.50)	AA	6 (0.38)	AG	5 (0.31)	GG	5 (0.31)	A	18 (0.53)	G	16 (0.47)
	$I1Z_{513}$			G-	2 (1.00)	--	2 (0.13)	G-	1 (0.06)	GG	13 (0.81)	-	5 (0.15)	G	29 (0.85)
I2	$I2Z_{792}$	A-	1 (0.50)	G-	1 (0.50)	AA	6 (0.38)	AG	3 (0.19)	GG	7 (0.44)	A	16 (0.47)	G	18 (0.53)
	$I2Z_{797}$	A-	2 (1.00)			AA	13 (0.81)	AC	1 (0.06)	CC	2 (0.13)	A	29 (0.85)	C	5 (0.15)
	$I2Z_{801}$	--	2 (1.00)			--	14 (0.88)	C-	0	CC	2 (0.12)	-	30 (0.88)	C	4 (0.12)
	$I2Z_{871}$	A-	2 (1.00)			AA	12 (0.75)	AG	3 (0.19)	GG	1 (0.06)	A	29 (0.85)	G	5 (0.15)
	$I2Z_{926}$	A-	1 (0.50)	C-	1 (0.50)	AA	6 (0.38)	AC	6 (0.38)	CC	4 (0.25)	A	19 (0.56)	C	15 (0.44)
	$I2Z_{937}$	G-	2 (1.00)			GG	8 (0.50)	GT	5 (0.31)	TT	3 (0.19)	G	23 (0.68)	T	11 (0.32)
I3	$I3Z_{1100}$	A-	1 (0.50)	G-	1 (0.50)	AA	10 (0.52)	AG	6 (0.32)	GG	3 (0.16)	A	27 (0.68)	G	13 (0.33)
	$I3Z_{1275}$			G-	2 (1.00)	AA	1 (0.06)	AG	2 (0.13)	GG	13 (0.81)	A	4 (0.12)	G	30 (0.88)
I4	$I4Z_{1551}$	C-	2 (1.00)			CC	12 (0.75)	CT	3 (0.19)	TT	1 (0.06)	C	29 (0.85)	T	5 (0.15)
	$I4Z_{1558}$			T-	2 (1.00)	GG	2 (0.13)	GT	5 (0.31)	TT	9 (0.56)	G	9 (0.26)	T	25 (0.74)
E5	$E5Z_{1851}$ (aa185)	C-	2 (1.00)			CC	13 (0.81)	CT	2 (0.13)	TT	1 (0.06)	C (Tyr)	30 (0.88)	T (Tyr)	4 (0.12)

[a] number of animals and genotype frequencies (%) in brackets.

[b] number of alleles and allele frequencies (%) in brackets.

Site: $5'Z_{(-326)}$ – 5'-region at *GH2-Z* copy, position 326 upstream from the transcription starting site; U – UTR; E – exon; I – intron; *Z* – *GH2-Z* copy, e.g., $5UZ_{288}$ – 5'-UTR at the *GH2-Z* copy position 288 according to the published o*GH* gene sequence (Orian *et al*., 1988; GenBank accession no. X12546); aa – amino acid number of the mature protein (considering N-terminal alanine as amino acid 1 which corresponds to amino acid 27 of the published oGH sequence with GenPept accession number CAA31063) (Orian *et al*., 1988).

Table IV-18. *Endonucleases*[1] *that discriminate SNPs found by SSCP analysis or DNA sequencing along the o*GH *gene copies.*

Copy	Site	Base substitution	Endonucleases that recognize new restriction site
GH1* or *GH2-N	**E1*N*$_{301}$**	t→a	*Alu*I
	I1*N*$_{480}$	g→a	*Ssp*I
	E2*N*$_{651}$	c→t	*Bfa*I, *Mae*I
	E2*N*$_{666}$	t→c	*Hpa*II, *Msp*I
	E3*N*$_{973}$	a→t	*Eco*47III
	E3*N*$_{1047}$	a→g	*Hae*III, *Hpa*II, *Msp*I
	I3*N*$_{1254}$	c→t	*Sal*I, *Taq*I
	I3*N*$_{1254}$	t→c	*Hae*III
	I3*N*$_{1283}$	a→g	*Bst*UI, *Fnu*DII, *Ksp*I, *Mvn*I, *Sac*II, *Tha*I
	E5*N*$_{1872}$	a→g	*Acc*III, *Bse*AI, *Bsp*EI, *Hpa*II, *Msp*I, *Mro*I
	E5*N*$_{1938}$	t→c	*Alu*I, *Pvu*II
	3U*N*$_{2050}$	c→g	*Dpn*I, *Dpn*II, *Mbo*I, *Nde*II, *Sau*3AI
	3U*N*$_{2069}$	g→c	*Alu*I
GH2-Z	**E2*Z*$_{644}$**	a→g	*Cfo*I, *Hha*I, *Hin*PII, *Kas*I, *Nar*I
	E2*Z*$_{704}$	c→g	*Bbr*PI, *Mae*II, *Pma*CI, *Pml*I
	E2*Z*$_{712}$	a→g	*Alu*I, *Pvu*II,
	I2*Z*$_{793}$	a→g	*Aos*I, *Avi*II, *Cfo*I, *Fsp*I, *Hha*I, *Hin*PII
	I2*Z*$_{937}$	t→g	*Pvu*II
	E3*Z*$_{1047}$	a→g	*Hae*III, *Hpa*II, *Msp*I
	I4*Z*$_{1551}$	t→c	*Bbr*PI, *Mae*II, *Nla*III, *Pma*CI, *Pml*I
	3U*Z*$_{2030}$	c→t	*Mse*I, *Tru*9I
GH1 or GH2-N vs. GH2-Z	**E2*N*$_{668}$→E2*Z*$_{668}$**[2]	g→(c or t)	*Hae*III, *Hpa*II, *Msp*I

[1] Only enzymes with palindromic/non-ambiguous recognition sequences.
[2] Differences between *GH2-N* (or *GH1*) copy and *GH2-Z* copy (SNP that allows distinguishing *Gh1*/*Gh1* animals from *Gh1*/*Gh2* or GH2/*Gh2* animals).

PCR-SSCP and sequencing techniques have some limitations for routine SNPs genotyping. PCR-SSCP needs a great effort in conditions optimization and can sometimes lead to misleading results. Although sequencing has become cheaper in recent years it needs several purification steps and results are rather difficult to interpret when DNAs from both chromosomes are analysed simultaneously (generated directly from a PCR) and insertions/deletions are present in one of them. The use of RFLP analysis for SNPs genotyping is an interesting option, because it is easy to apply and allows to genotype exactly the analysed animal. Thus, SNPs disclosed by PCR-SSCP (**Table IV-4**) and sequencing analyses (**Table IV-15**) were examined to find restriction sites that could be recognized by endonucleases useful for RFLP analysis. A total of 13 SNPs within *GH1* or *GH2-N copies*, eight SNPs within *GH2-Z* copy and one SNP that allows allows distinguishing *GH1* or *GH2-N copies* from *GH2-Z* copy

are listed in **Table IV-18**. The remaining SNPs need to be genotyped using alternative techniques as allele-specific PCR or SNaPshop.

IV.4 Transcription factors binding sites

The characterization of the 5'-region (1 kb upstream the transcription starting site) of the sequences GenBank accession numbers DQ450146 (*GH1* copy), DQ461666 (*GH2-N* copy) and DQ461642 (*GH2-Z* copy) disclosed differences between copies in the putative transcription factors binding sites (**Figure IV-9**; see **Appendix 4** for detailed information on site positions and sequences). It is evident from the **Figure IV-9** that *GH1* and *GH2-N* copies have similar putative binding sites and both copies differ considerably from the *GH2-Z* copy. Of particular interest are the different localization of POU1F1 binding sites between the copies; the inexistence in the *GH2-Z* copy of a putative binding site for Zn-15 which is known to enhance *GH* gene expression when in association with POU1F1; the lower number of YY1 binding sites in *GH2-Z* copy; and the existence of several putative binding sites for STAT.

The high divergence found in the regulatory region of the o*GH* copies concurs to copies being expressed in different tissues, i.e., *GH1* and *GH2-N* in pituitary and *GH2-Z* in placenta (Lacroix *et al*., 1996, 1999; Sartin *et al*., 1996).

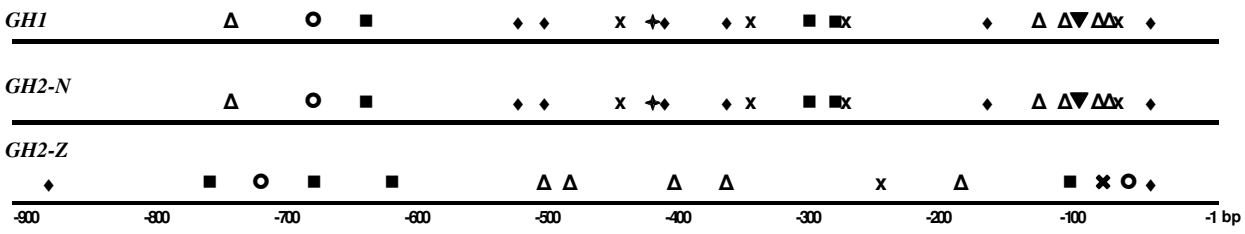

Figure IV-9. *Putative transcription factors binding sites at the 5'-region at* GH1*,* GH2-N *and* GH2-Z *copies.*

Δ - POU1F1, POU1F1a, POU1F1b and/or POU1F1c (pituitary-specific transcription factor 1); ✖ - Ap-1 (activator protein 1); ✪ - AP-2 and NF-1, AP-2alphaA and AP-2alphaB (activator protein 2) and nuclear factor 1; ✦ - GR (glucocorticoid receptor); ♦ - T3R and/or T3R-alpha (3,5,3'-triiodothyronine receptor); ■ - STAT1, STAT5A, STAT5B and/or STAT6 (signal transducer and activator of transcription); ▼ - Zn-15 (zinc finger protein); **x -** YY1 (yin and yang factor 1).

IV.5 Protein structure prediction

The protein structure analysis comparison between GH1, GH2-N and GH2-Z was performed by using the the PROSITE SCAN package tools (http://us.expasy.org/) from the ExPASy Molecular Biology Server.

All the analysed protein variants have a GH/PRL and related hormones signature 1 (motif CfSetIpAPtgkneAqqksdleLlrisllLiqSW) and 2 (motif CFRkDLhkTetYlrVmkC) at positions 53-86 and 164-181, respectively. Tyrosine kinase phosphorylation site (motif Kef.Ert.Y at position 30-36) and luecine zipper pattern (motif LlknyglLscfrkdLhktetyL at position 155-176) are present also in all the protein variants analysed. Amino acid substitution originate differences in the number of N-myristoylation, protein kinase C phosphorylation, casein kinase II phosphorylation and N-glycosylation sites found at each of the oGH copies (**Table IV-19**). All GH2-N protein variants have a N-myristoylation site (motif GLfaNA) at positions 9-14, but this site is absent in the GH2-Z protein variants in which G^9 is substituted by C^9 or R^9 (**Table IV-5**).

Table IV-19. *Protein motif comparison between several oGH protein variants.*

Location*	Motif	V_NA	$V_N(S^{32})^a$	V_ZA	$V_Z(G^{63})^b$	$V_Z(G^{63},N^{64})^c$	$V_Z(S^{160})^d$
N-myristoylation site							
9 – 14	GLfaNA	**x**	**x**	**–**	**–**	**–**	**–**
40 – 45	GQrySI	**x**	**x**	**x**	**x**	**x**	**x**
63 – 68	GNneAQ	**–**	**–**	**–**	**–**	**x**	**–**
160 – 165	GLlsCF	**x**	**x**	**x**	**x**	**x**	**–**
Protein kinase C phosphorylation site							
28 – 30	TfK	**x**	**x**	**x**	**x**	**x**	**x**
32 – 34	SeR	**–**	**x**	**–**	**–**	**–**	**–**
62 – 64	TgK	**x**	**x**	**x**	**x**	**–**	**x**
106 – 108	SdR	**x**	**x**	**x**	**x**	**x**	**x**
131 – 133	TpR	**x**	**x**	**x**	**x**	**x**	**x**
Casein kinase II phosphorylation site							
28 – 31	TfkE	**x**	**x**	**x**	**x**	**x**	**x**
63 – 66	SknE	**–**	**–**	**x**	**–**	**–**	**x**
71 – 74	SdlE	**x**	**x**	**x**	**x**	**x**	**x**
ASN_glycosylation site							
158 – 161	NYSL	**–**	**–**	**–**	**–**	**–**	**x**

[a] - V_NH; [b] – V_ZD and V_ZE; [c] – V_ZJ; [d] - V_ZC, V_ZG and V_ZH (see **Table IV-6**).

V.

DISCUSSION

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'.”

Isaac Asimov (1920 - 1992)

V.1 Animals and milk records

The main objective of the present work was to detect polymorphisms at the o*GH* gene in "Serra da Estrela" animals reared under the "traditional" production system. As each shepherd practices his own system according to the genetic potential of his animals, the feed availability at the farm at each moment and other management decisions related with individual flocks' constraints at each moment, the present study was extended to seven flocks. All the animals present in each flock were sampled for blood regardless whether they were related or not. This kind of approach may have limited the level of polymorphism found in this study. However this could have been in part overcome by the fact that the analyses were performed in seven flocks. Rams from ANCOSE Male Testing Centre were also genotyped. These rams came from different flocks spread all over the "Serra da Estrela" region and are sons of the best yielding ewes, thus pre-selected as it often happens in studies seeking for genetic markers for milk production (Parmentier *et al*., 1999), not randomly chosen as it is mandatory in genetic diversity studies.

The seven flocks were chosen according to the number of ewes (50 or more), breed variety (white or black in a proportion similar to the one of the overall population) and milk yield levels (low, medium and high). The mean number of ewes in "Serra da Estrela" flocks is low. However flocks with a lower number of animals were not chosen because it would impair the statistical analysis.

Answers to the production system questionnaire suggest that the production system changed little since it was thoroughly characterised in 1993 (Gulbenkian *et al.*). Minor differences could perhaps be related with the high number of animals in each flock and with the sanitary legislation related to brucellosis control that restricted the use of communitary barren lands.

V.2 oGH copy number genotypes

Duplication of the o*GH* gene has been described in sheep (Valinsky *et al.*, 1990), in goat (Yamano *et al*., 1991) and in humans (Chen *et al.*, 1989), but not in bovine. Allele *Gh1* (not duplicated) was found to be rare (10%) in "Serra da Estrela" population as it was observed also in Assaf, Awassi and Merino (Valinsky *et al*., 1990), and in "Merino da Beira Baixa" (I.C. Santos, personal communication) ovine breeds. Moreover, in ovine breeds, animals carrying

the *GH2-Z* copy (*Gh2* allele) represented approximately 90% of the population suggesting that it may confer some selective advantage. Moreover, as duplication confers tissue- and stage-specific activity to the *GH2-Z* gene, amino acid divergence between the o*GH* copies was possibly caused by positive Darwinian selection as suggested by Ohta (1993).

V.2.1 Implications in milk yield

The number of studies analysing the biological effect of o*GH* copy number are scarce. Nevertheless, it was investigated in ram lambs selected for body composition and GH levels (lean and fat lines). Although *GH* copy number genotypes had no effect on GH secretion parameters or body composition, it was observed that they significantly influenced the response to GHRH (Gootwine *et al*., 1998) suggesting that the natural occurring variation in o*GH* copy number influence GH axis response to different nutritional and perhaps physiological status. Other studies reported that the effects of the o*GH* copy number in transgenic animals expressing an additional o*GH* gene copy were an increased growth rate and a decreased body fat content (Adams *et al*., 2002). The effects of the naturally occurring variation in o*GH* copy number have been studied in Awassi dairy sheep (Gootwine and Ofir, 1996). The progeny of two *GH1/GH2* rams were studied showing that *GH1/GH2* daughters of only one of them yielded significantly more milk per lactation than their *GH2/GH2* half sib (513 l *vs* 465 l).

"Serra da Estrela" ewes that received the *Gh2* allele from their progenitor yielded 21.9 ± 7.5 l/150 d more milk than ewes that received the *Gh1* allele (model 2; **Figure IV-4**). The higher milk yield observed in ewes with increasing number of *Gh2* alleles points to a possible dominant effect of *Gh2* allele.

The *GH2-N* is expressed in anterior pituitary gland somatotrope cells (Warwick *et al.*, 1989). As the *GH2-N* and *GH2-Z* gene copies are regulated differently, *GH2-Z* is not expressed in the pituitary (Gootwine *et al*., 1996). It is expressed in placenta trophoblasts and syncitium between day 30 and 60 of gestation, a period of maximum placental growth (Lacroix *et al.*, 1996; 1999). Therefore, during early gestation, animals carrying *Gh2* allele have two sources of circulating GH: the pituitary GH derived from *GH2-N* copy expression and the placental GH derived from *GH2-Z* copy expression. Hence, it is reasonable to hypothesise that *Gh2* carrying ewes have higher levels of GH which might influence directly placental and mammary gland development in the early gestation, and indirectly mammary cell differentiation in the last phase of gestation through higher levels of placental PRL.

V.2.2 Transcription factors binding sites and o*GH* copies regulation

The analysis of 1 kb fragment upstream from the start of transcription binding site, revealed that *GH1* and *GH2-N* copies have identical promoter sequences, divergent from *GH2-Z* promoter (**Figure IV-9**). Of particular importance in transcription regulation are the binding sites located between positions -300 to -1. This region presents at *GH1* and *GH2-N* copies four POU1F1 binding sites and a Zn-15 binding site between them; the proximal POU1F1 binding site interacting with an YY1 binding site. This group of binding factor sites is flanked by two T3R response elements. Putative binding sites for STATs were predicted within the -300 to -250 region, flanked proximally by an YY1 putative binding site. The T3R proximal element is the only one common to the three *GH* copies. At *GH2-Z* copy, one binding site for each AP-1, AP-2, STATs, POU1F1 and YY1 transcription factors was predicted. In **Figure IV-9** and in **Appendix 4**, only putative binding sites similar to those described in *GH*, *PRL* and chorionic somatomammotropin genes in human and rat were listed. However a considerable higher number of transcription factors binding sites were predicted: among them are several GR, Sp1 and nuclear factor-I (NF-1) binding sites. The differences in the localisation of the observed putative binding sites are in agreement with the differential activation of GH and chorionic somatomammotropin gene expression by Sp1 and T3R (Tansey and Catanzaro, 1991). In addition, the presence of an AP-2 binding site at position -64 of the *GH2-Z* copy might mediate *GH2-Z* copy expression in the placenta in a similar way to the AP-2 mediated trophoblast-specific transcription of the oPL gene (Liang *et al*., 1999).

To elucidate the effective role played by the predicted binding sites in regulation of o*GH* gene copies, DNase I protection analysis, electrophoretic mobility-shift assays and mutational analysis should be performed in the future.

*V.3 PCR-SSCP polymorphisms at the o*GH *gene*

The high level of polymorphism detected in each o*GH* gene copy and between o*GH* gene copies confirms the PCR-SSCP as an efficient technique to detect mutations (**Table IV-1** and **Figure IV-5**), as it has been shown in other studies in sheep (Bastos *et al.*, 2001; Santos *et al*., 2004), in goats (Malveiro *et al.*, 2001; Marques *et al.*, 2003), in cattle (Lee *et al.*, 1994b; Lagziel *et al*., 1996; Yao *et al.*, 1996; Hines *et al.*, 1998; Lagziel and Soller, 1999) and in pigs (Kaminski and Wachek, 2002). Moreover, it is cheaper and faster than other techniques like

allele-specific PCR or RFLP. Nevertheless, it has limitations such as the occasional appearance of extra bands that may complicate the analysis specially when analysing a duplicated gene.

Up to seven alleles were discriminated by SSCP in the analysed o*GH* fragments (**Table IV-1**), some of them present at low frequencies in the studied flocks. Fragments III, IV, VI and VII are illustrative of the high variability of the o*GH* gene in "Serra da Estrela" breed. The high number of SSCP patterns found was somehow expected since the analyses were performed simultaneously on the two copies of the o*GH* gene.

The optimal conditions for the PCR-SSCP analysis, once settled, can be used to genotype for known mutations and to screen for new ones simultaneously, being for that reason, very popular in cancer research (Berggren *et al*., 2000; Frayling, 2002; Kringen *et al*., 2002) and in monitoring microbial communities (Albuquerque and Costa, 2003; Anderson and Cairney, 2004; Etchebehere *et al*., 2003). The application of PCR-SSCP to detect polymorphism in animals has increased in the last years and it probably will continue to increase concomitantly with the emergence of alternative ways to perform the SSCP analysis (Ganguly, 2002) such as capillary electrophoresis-based SSCP (Andersen *et al*., 2003; Larsen *et al*., 1999) or fluorescent-SSCP (Scholl *et al*., 2001) for instance.

SSCP patterns were found to be present at different frequencies in each flock (**Table IV-1**). In fragments I, II and V, pattern A is largely dominant over the others (frequency higher than 90%), namely in the high milk yielding flocks, what suggests that through traditional selection, unfavourable alleles were eliminated, thus possibly changing population HWE.

Results from HWE tests (**Table IV-3**) showed deviations from the expected values in all but fragments II_N and III_N. It is interesting to notice that for the same fragment in copies *GH2-N* and *GH2-Z*, the deviation from HWE seems to be due to different causes. In fragments III_N, IV_N and VI_Z deviation from HWE is mainly due to significant levels of heterozygote excess which might indicate the presence of overdominant selection or the occurrence of outbreeding. However, in fragments VII_N and III_Z, IV_Z and VII_Z HWE deviation results from a homozygote excess what could indicate the occurrence of one or more of the following four events: 1) those fragments were under selection; 2) a "null allele" could be present leading to a false observation of homozygote excess what is most likely to be the case in *GH2-Z* fragments; 3) high levels of inbreeding could have occured; 4) the population could be substructured leading to Wahlunds' effect (Dorak, 2006). Indeed, *Gh1*/*Gh2* animals, if they result from a *Gh1*/*Gh1* x *Gh1*/*Gh2* mating, are identical to their *Gh1*/*Gh2* parent at *GH2-Z* copy and thus

their inbreeding coefficient is probably higher increasing artificially the homozygote excess in the *GH2-Z* copy fragments.

V.3.1 Molecular characterization of the SSCP patterns

As *GH1*, *GH2-N* and *GH2-Z* sequences are highly homologous it was not possible to design specific primers for the exons and the 3'-UTR of each copy. However, the approach described in the present study (**section IV.2.3**) allowed the SSCP patterns to be interpreted and *GH2-N* and *GH2-Z* copies genotypes attributed.

Valinsky *et al.* (1990), Gootwine *et al*. (1993; 1996) and Ofir and Gootwine (1997) reported three *Pvu*II-RFLPs related to the o*GH* copy number, located at position 712 in exon 2 (restriction site present only in the *GH2-N* copy), position 932-937 in the intron 2 (restriction site mutated in the *GH2-Z* copy) and position 1935-1939 in the exon 5 (restriction site present both in the *GH2-N* and *GH2-Z* copies). The existence of polymorphisms at *Pvu*II restriction sites in exons 2 (*GH2-N* copy) and 5 (*GH2-Z* copy), identified in the present work, excluded the use of *Pvu*II-RFLP to discriminate between o*GH* copies (**Table IV-4**); this could be achieved using PCR-SSCP analysis of exon 2 as described (**section III.4**).

V.3.1.1 oGH gene polymorphisms

Results presented in **Table IV-4** and **Table IV-5** revealed copy specific polymorphisms (24 at the *GH2-N* and 14 at the *GH2-Z* copies) and a common polymorphism at position 1047. Alignment of sequences of the GH2-N and GH2-Z predicted mature proteins from all the genotyped sheep exhibiting the two o*GH* gene copies (17 *Gh2*/*Gh2* and two *Gh1*/*Gh2* animals), revealed that the protein copies always differ at two amino acids (aa): aa(-7) (P $\rightarrow$ L; site 624) and aa9 (G $\rightarrow$ R or C; site 668) as reported by Wallis *et al*. (1998). Aa63 differs also between the predicted protein copies (G $\rightarrow$ S; site 1057) in most animals; however, in some, G^{63} is present in both GH2-Z and GH2-N copies. GH1 protein sequence was found to be identical to the GH2-N consensus sequence (Orian *et al*., 1988) in accordance with the finding by Ofir and Gootwine (1997) in Awassi, Romanov and Romney ewes.

The present (**Table IV-1**, **Table IV-4**, **Table IV-5** and **Figure IV-5**) and previous results in other Portuguese ovine breeds (Bastos *et al*., 2001; Santos *et al*., 2004), as well as in Assaf breed (Santos *et al*., unpublished results), in Israeli breed (Ofir and Gootwine, 1997) and in Indian breeds (GenBank accession numbers: DQ166369-74 and DQ176733-47) showed that coding regions are much more polymorphic in ovine than in bovine *GH* gene. In bovine, polymorphisms detected by SSCP analysis are present mainly in non-coding regions and in

exon 5 (Falaki *et al*., 1997; Lagziel and Soller, 1999; Ge *et al*., 2003). The low level of polymorphism observed in the 5'-UTR and in exon 1 (fragments I and II, respectively) is in agreement with previous findings indicating that these regions are highly conserved in *GH1* and *GH2-N* genes in ovine (Ofir and Gootwine, 1997). Exon 2 was the most polymorphic one in "Serra da Estrela" ewes and also in Merino da Beira Baixa (Santos et al., 2004), but not in caprine breeds (Malveiro *et al*., 2001; Marques *et al*., 2003). This suggests that those polymorphisms arose after species differentiation. In exon 4, no polymorphism was revealed in spite of the two observed SSCP patterns, suggesting that DNA modification(s) other than nucleotide mutations could be detected by SSCP analysis. Conversely, eight variants were described in exon 4 (GenBank accession numbers: DQ176740-47) in Indian ovine breeds, mainly within the exon 4-intron 4 junction region, and a high polymorphism level was also observed in caprine breeds (Malveiro *et al*., 2001; Marques *et al*., 2003).

Many of the polymorphic sites detected by SSCP analysis were located near to CCC or AAA repeated regions suggesting that SSCP analysis might bias SNP detection towards repeated sequences with low secondary structure of the single strand DNA being less efficient in mutation dectetion within complex local secondary structures as palindromic regions, as observed in bovine and humans (Barendse and Armitage, 2001).

Taking fragment genotypes as a starting point, o*GH* haplotypes were inferred in both copies for some genotypes, but it was not possible to determine the phase for all genotypes, so statistical analyses could not be performed involving haplotypes as it has been done in bovine by Lagziel *et al*. (1996). At the time of blood collection many of the ewes' sires were no longer in the flocks, and therefore could not be genotyped. Nevertheless, considering pedigree information of 750 animals, a large number of oGH haplotypes was inferred, hence revealing considerable genetic diversity at the o*GH* gene within this breed. Eighty six haplotypes were inferred in the *GH2-N* copy, and 32 in the *GH2-Z* copy which is much more than the 14 different haplotypes reported by Lagziel *et al*. (1996) in Israel Holstein bovine breed.

V.3.1.2 Predicted protein variants

The genetic polymorphisms detected in both copies could either involve regulatory regions and affect gene transcription rate (Pesole *et al*., 2000; discussed in **section V.2.2**), or influence the secondary structure of the protein and its post-transcriptional function (Woychik *et al*., 1984), or be linked to a genotype of interest located elsewhere (Sneyers *et al*., 1994).

Predicted protein variants are listed in **Table IV-6**. Considering the predicted amino acid changes between GH2-N protein variants, aa(-16) is part of the GH signal peptide, aa3 is

near the GH NH_2-terminal, amino acids 8, 22, 25 and 32 are in helix 1, with aa8 being involved in the second receptor-binding site as defined by Cunningham *et al*. (1991) for hGH. The remaining amino acids involved in variant differentiation are between helices 1 and 2.

Considering the predicted GH2-Z protein variants, aa(-1) is part of the GH signal peptide, aa2 is near the GH NH_2-terminal, amino acids 9, 21 and 23 are in helix 1, with aa9 being part of the second receptor-binding site, amino acids 55, 59, 63 and 64 are between helix 1 and 2, with aa63 being part of the first receptor-binding site, and aa160 is between helix 3 and 4. NH_2-terminal residues, involved in the GH receptor binding, have a substantial galactopoietic activity in cows (Eppard *et al*., 1992). Hence, polymorphisms found in this region could account for the differences observed in the milk yield.

Several amino acid substitutions found within and/or between GH copies were predicted to change the nature/number of protein's motifs (**Table IV-19**) that might influence protein structure, stability, or activity:

- The changes at position 9 (G to R in GH2-N or to C in GH2-Z) disrupt the N-myristoylation site. The possible link between a fatty acyl group and GH could mediate protein-protein interactions or change the structure and activity of the protein to which it binds (Voet *et al*., 1999). S^{160} GH2-Z variants (V_ZC, V_ZG, and V_ZH) have also the N-myristoylation site disrupted, and inversely the VzJ (G^{63},N^{64}) variant presents an extra N-myristoylation site.
- Several predicted GH variants could be differently regulated as they were found to: either have one additional PKC phosphorylation site (S^{32} in V_NH) or lack one (G^{63},N^{64} in V_ZJ) as compared with V_NA and V_ZA variants, respectively; and/or lack one casein kinase II phosphorylation site (VzD and V_ZJ) with regard to the V_ZA variant.
- The occurrence of a putative N-glycosylation site in V_ZC, V_ZG, and V_ZH variants and the presence of the appropriate oligosaccharide-processing enzymes could lead to the cotranslational N-glycosylation of GH (Voet *et al*., 1999). It is not known whether it affects the biological function of the protein or not.

Some authors report that GHR processes great conformational plasticity to bond with dissimilar sites on GH, thus an amino acid substitution *per si* possibly does not disrupt GH/GHR binding (Sami *et al*., 1999). However, GH myristoylation, phosphorylation and/or glycosylation states might reduce the GH/GHR binding affinity altering GH-signal transduction and consequently its biological activity.

The present work disclosed new oGH variants coded by both gene copies. To elucidate the influence of the underlying amino acid changes on the structure-function of the GH further studies are needed. One possible approach could be the heterologous production of functional GH variants as suggested by Appa Rao *et al*. (1997).

V.4 Milk yield and composition

Recent studies performed in goats (Malveiro *et al*., 2001; Marques *et al*., 2003) and cattle (Lagziel *et al*., 1996; Lagziel and Soller, 1999; Yao *et al*., 1996) allowed the establishment of associations of GH SSCP patterns with milk traits.

In the present work significant associations were found between o*GH* genotypes (**Table IV-7**, **Table IV-8** and **Table IV-9**) or phenotypes (**Table IV-10**, **Table IV-11** and **Table IV-12**) and milk production in "Serra da Estrela" ewes. Some of the alleles uncovered by SSCP and sequencing analyses were not found to correspond to homozygous genotypes (and their corresponding phenotypes). This limited the statistical analysis to the use of a model where only additive genetic effects were considered.

Considering the overall population, differences of approximately 21 l/150 d ($P<0.05$) were observed between the most and the least productive genotypes either for *GH2-N* (N7 *vs.* N5; **Table IV-7**) or *GH2-Z* copies (Z7 *vs.* Z2; **Table IV-8**). However, milk yield differences reach 31.4 ± 9.0 l/150 d between the extreme productive GH2-N phenotypes [AB_N *vs.* (AH_N and /or BE_N); **Table IV-10**], and only 16.6 ± 8.3 l/150 d between the extreme productive GH2-Z phenotypes [BD_Z *vs.* (AD_Z and/or BE_Z); **Table IV-11**]. The results suggest that selecting for the highest milk yielding GH2-N phenotype would maximise the selection response.

At the *GH2-N* copy, the least (N5) and the most (N7) productive GH2-N genotypes code for the same protein variant. Therefore, the observed effect upon milk yield could be due to the efficiency of mRNA processing or to the stability of its pre-processed form.

In the *GH2-Z* copy, the Z2 genotype codes to a protein variant with the following amino acids: F^{2},R^{9},L^{21},S^{63},K^{64},G^{160}. It corresponds to the published oGH sequence number Q95205 (Lacroix *et al*., 1996) and differs in one amino acid from the one coded by the Z7 genotype that has a C in position 9 (C^{9}). The effect of an arginin at position 9 was discussed by Wallis *et al*. (1998) who suggested that a R^{9} variant can have increased binding affinity to GHR binding site 2 as it enables a salt bridge formation with E^{122} and hydrogen bond to S^{120} of the GHR.

Whether the Arg→Cys substitution influences GH binding to its receptor or protein folding remains to be elucidated. In any way, the genotypes which code for C^{9} variant were positively ($P<0.05$) associated with milk yield (**Table IV-9**).

The phenotypes AB_N *vs.* (AH_N and /or BE_N), corresponding to the GH2-N copy, are the most and least productive ones respectively; they differ from each other in aa(-16) and aa32. The Leu→Pro substitution at aa(-16) introduces probably a kink in the GH signal peptide in variants V_NE and V_NH (both with P^{-16}), which might impair their binding to the signal recognition particle and reduce GH secretion rate. As a result, animals carrying the P^{-16} protein variant could have reduced circulating GH level and consequently lower milk yielding aptitude as suggested by the present results. Additionally, a Phe→Ser substitution at aa32 located in helix 1 creates an additional PKC phosphorylation site that might interfere with V_NE and V_NH protein folding.

The phenotypes BD_Z *vs.* (AD_Z and/or BE_Z), corresponding to the GH2-Z copy, are the most and least productive ones respectively; they differ from each other in aa9 and aa63. BD_Z is homozygous for C^{9}, and AD_Z and BE_Z are heterozygous phenotypes in which variants V_ZA and V_ZE present an Arg at position 9 and variants V_ZB and V_ZD carry a Cys at position 9. The putative influence of these amino acid changes were discussed above. The three phenotypes are heterozygous for aa63. Aa63 belongs to the binding site 1 in human GH (de Vos *et al*., 1992). Wallis *et al*. (1998) suggest that the substitution of S^{63} together with an Arg at position (R^{9}) at the GH2-Z copy (V_ZA) could inhibit GH-GHR binding. The presence of the G^{63}, described for the first time in ovine populations in the present work, and its positive effect on milk yield is in agreement with that assumption. In nature, although acting at different stages, both oGH copies act to control ewes' metabolism. The GH2-N copy contribute to the homeorhetic control of the metabolism throughout life (Bauman and Currie, 1980) and GH2-Z copy is thought to influence placental and foetal growth, contributing to successful gestations of twins (Lacroix *et al*., 1999). Whether the GH2-Z copy influences directly milk yield, or not, remains controversial. However, significant interactions between GH2-N and GH2-Z were observed (**Table IV-9** and **Table IV-12**) indicating that some epistatic effects may occur between GH copies, i.e., the effect of one copy depends on the other copy genotype/phenotype. Considering both copies simultaneously, the milk yield differential between (N2+Z7) and (N5+Z2) genotypes was 39.6 ± 7.5 l/150 d (**Table IV-9**) and the differential between (AA_N+BB_Z) and [(AH_N and/or BE_N)+BB_Z] phenotypes was only 16.2 ± 8.1 l/150 d (**Table IV-12**). Thus, the apparent joint effect of *GH2-N* and *GH2-Z* genotype could improve milk yield in 25% as

compared with mean milk yield of the analysed population. Within each flock, the positive influence of the combined genotypes on milk yield ranged from 10% in FL2 to approximately 35% in FL3, FL5 and FL6.

The effect of genotypes/phenotypes differences in milk yield changes from flock to flock. In fact, one genotype/phenotype presenting the highest milk yield within one flock could be not the best in other flock. The results suggest that genotype/phenotype x environment interaction might influence the expression of milk yield potential in ewes as it was previously described in "Serra da Estrela" ewes (Carolino *et al*., 2003) and in Sarda ewes (Sanna *et al*., 2002). Nevertheless, the ranking of genotypes/phenotypes in each flock according to their effects on milk yield showed that those which influence milk yield positively within a flock have an expected similar effect in the other flocks and the same occurs for the low yielding genotypes/phenotypes. However, exceptions can occur as observed in FL5. Thus, it is important to test genotypes x environement (i.e., flock) interaction.

The influence of oGH genotypes/phenotypes were also tested on milk yield composition (**Table IV-13**, **Table IV-14** and **Table IV-15**). Ewes with associated *GH2-N*+*GH2-Z* genotype N2+Z7 more milk with higher milk fat content and similar protein content than ewes with genotype N5+Z2. Moreover, a similar observation was made for AA_N+BB_Z protein phenotype.

In general, the oGH genotypes/phenotypes with higher milk yield potential influenced positively milk fat and protein content, and milk fat, protein and fat+protein yields. This is an interesting result because usually, increasing milk yield results in lower fat and protein contents. However further studies should be performed to confirm the present results, because only one year of milk composition records was consider. Nevertheless, previous studies reported a positive effect of GH polymorphisms in milk fat content (Khatami *et al*., 2005; Lee *et al*., 1994a). The higher milk fat content observed could result form the enhanced availability of milk fat precursors due to GH-induced increase in adipose tissue lipolysis.

V.5 Single nucleotide polymorphisms (SNPs)

In this work, the o*GH* copies were sequenced to characterise SSCP alleles. The procedure followed adopted, in addition to the detection of polymorphisms not identified by SSCP, to screen intronic regions for SNPs. SNPs were found to be more frequent in intron 1 at the *GH2-N* copy, and in the 5'-region and in intron 2 at the *GH2-Z* copy. It was also found a

SNP (synonymous mutation) in the exon 5 at the *GH2-Z* copy not detected by SSCP analysis (**Table IV-16** and **Table IV-17**).

SNPs found in the intronic regions were expected to be in HWE, but some were not. Deviation from HWE could be due to selection as well as to other factors discussed in **section V.3**. Deviation of HWE towards homozygote excess suggests that selection could have been applied to sites 5'$N_{(-125)}$, I2N_{793} and I3N_{1259}. Site 5'$N_{(-125)}$ is located at the *GH1* and *GH2-N* promoter regions between Sp1 and POU1F1 binding sites (position -125; **Figure II-4**), within an important region for o*GH* gene transcription regulation where SNPs have been shown to affect IGF-1 concentration in cattle (Ge *et al*., 2003). SNPs in intronic regions of several genes from the somatotropic axis have been shown to influence productive traits: e.g., a SNP in intron 3 of the *GH* gene influenced milk fat yield in Holstein cows (Lee *et al*., 1994a) and a SNP in intron 5 of POU1F1 influenced milk and protein yields, fat percentage, body depth, angularity, and rear leg set in Italian Holstein-Friesian bulls (Renaville *et al*., 1997). Homozygote excess was also observed for several SNPs at the *GH2-Z* copy.

Due to the insufficient number of animals screened, statistical analyses were not attempted to search for associations between SNPs and milk production traits. In **Table IV-18** a list of endonucleases is proposed to rapidly identify some of the SNPs found at the *oGH* in "Serra da Estrela" ovine breed through RFLP analysis.

V.6 Future perspectives:

In traditional quantitative genetics, selection is based on phenotypes and parental information whereas in molecular genetics, selection could be made directly upon a genotype via candidate gene marker approach.

An ideal dairy system would be the one where:

- all animals could be correctly identified (e.g., combining traditional identification systems with electronic identification such as reticulo-ruminal encapsulated chips);
- parental information is accurate (extensive use of artificial insemination, AI, and/or parentage testing) and available for all individuals; and
- phenotype data records are fully reliable (data normalization and validation).

Combining quantitative and molecular genetics (i.e., MAS) in a selection index where the best males are selected to sire the next generation should maximise genetic progress.

However, the "Serra da Estrela" traditional ovine production system is not an ideal one, namely because AI application is still limited. An alternative approach to improve the existing selection scheme would be to *GH* genotype solely lamb daughters of the best females within the flock and retain only those with the most production-relevant genotypes. Such an approach might reduce generation intervals and improve greatly the milk yield response in dairy ewes.

VI.

CONCLUSIONS

One never notices
what has been done;
one can only see
what remains to be done."

Marie Curie, letter to her brother (1894)

In conclusion, the *GH2-N* and *GH2-Z* genotypes were shown to significantly affect milk yield in the "Serra da Estrela" ewes. The apparent joint effect of *GH2-N* and *GH2-Z* genotypes could, on average improve milk yield adjusted to 150 d lactation on 25% as compared with mean milk yield of the analysed population.

The high level of polymorphism observed suggests that genetic variability at the *GH* gene in *Ovis aries* could be exploited to produce genotypes with increased genetic merit.

In addition the present results suggest that oGH genotypes/phenotypes influence significantly milk yield in sheep, without detrimental influence in milk composition. However, the use of the o*GH* gene as a marker for sheep selection requires a previous knowledge of the animals' behaviour within the flock where they are reared.

VII.

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APPENDICES

Appendix 1 Questionnaire for dairy sheep farmers

QUESTIONNAIRE FOR DAIRY SHEEP FARMERS

Date ____/____/_____

FARMER: _____
ANCOSE code _____
Address: _____
Place: _____
Total area = _____ ha Owned = _____ ha
Rented = _____ ha

SHEEP AND SHEEP PRODUCTS

	Ewes		**Cheese**		**Lambs weaned**		
	Primiparous	Multiparous	Maximum No.	Mouth	No.	Age	Weight
Sheep							

1) FARM HAND-LABOUR

Who does the shepherding? _____
There are other workers? _____
When? What they do? _____
Shepherd _____
Person(s) for milking _____
Person(s) for cheese making _____
Other _____

2) PASTURES

Do you have pastures?

Natural?	Owned	No	Yes	Area = _____ha
	Rented	No	Yes	Area = _____ha
Sown?	Owned	No	Yes	Area = _____ha
	Rented	No	Yes	Area = _____ha

Which crops do you seed? _____

Do you fertilize them? _____
When? _____
With what? _____

There is water for irrigation? Yes No Electricity Yes No

Do you irrigate the cultures?
How? _____

Which kind of vegetation do the ewes grass in? _____
Autumn _____
Winter _____
Spring _____

Are they under tree cover pasture? Natural? No Yes
Sown? No Yes

Which are the dominant shrub species? _____
Natural _____
Sown _____

At which time do the ewes go to the pasture and when do they return?
Autumn _____
Winter _____
Spring _____

How much time do the ewes spend till the pasture? _____
Autumn _____
Winter _____
Spring _____

Do you have pastures with fences or ewes grass all over the area?
Autumn _____
Winter _____
Spring _____

Do the lactating ewes grass separately from the remaining flock?
Autumn _____
Winter _____
Spring _____

Do the ewes grass the remains of other crops after harvesting? Which ones?
Autumn _____
Winter _____
Spring _____

Do the ewes grass in communitary barren lands? Do you practice transhumance?
Autumn _____
Winter _____
Spring _____

3) MILKING

In what month do you start milking? _____
In what month do you stop milking? _____
Which is the month with higher milk production? _____
Who milks the ewes? _____
How much time does it take? _____
Autumn _____
Winter _____
Spring _____
At which time are the ewe milked? _____
Autumn _____
Winter _____
Spring _____

4) SUPPLEMENTARAY FEED

Do you give any supplementary feed during milking? _____ (How many? What kind?)
Autumn _____
Winter _____
Spring _____

Do you give any supplementary feed in the pen?
Hay
Which kind? _____
How much do you give to each ewe?
Autumn _____
Winter _____
Spring _____
Locally produced or purchased? _____
Straw
Which kind? _____
How much do you give to each ewe?
Autumn _____
Winter _____
Spring _____
Locally produced or purchased? _____

4) MILK AND CHEESE

What do you do with milk? _____
How many litres are usually necessary to make a cheese? _____
Autumn _____
Winter _____
Spring _____
How much does a cheese weigh? _____
Autumn _____
Winter _____
Spring _____

Appendix 2 Flocks' average milk data records in the lactations of the years 95/96 and 96/97 *(36 flocks and 2520 ewes)*

Flock	No. Ewes	Lambing age (month)	Lambing date	number of lambings	Type of lambing	Suckled milk (l)	Milked milk (l)	Total milk yield (l)	Lactation days	Milk yield (l/150 d)
FL1	57	58.7	15-Out	4.9	1.4	28.6	88.9	117.5	224.3	90.0
FL2	67	47.0	17-Out	4.0	1.4	31.4	160.1	191.4	209.7	152.8
FL3	84	45.9	02-Nov	3.7	1.5	34.1	203.2	237.3	186.6	210.0
FL4	79	58.7	16-Out	4.9	1.4	27.9	85.2	113.1	225.5	85.4
FL5	148	39.4	26-Out	3.3	1.5	21.2	147.6	168.8	184.5	139.5
FL6	82	47.2	11-Out	4.0	1.5	35.7	179.2	214.9	207.0	179.5
FL7	48	39.5	11-Out	3.3	1.5	42.3	202.9	245.2	213.9	191.2
FL8	70	60.0	10-Out	4.9	1.6	35.9	117.6	153.5	214.4	122.2
FL9	79	58.3	06-Out	4.9	1.5	47.9	173.3	221.2	216.5	167.0
FL10	103	53.4	23-Set	4.6	1.3	38.5	108.4	146.9	204.7	113.2
FL11	60	41.6	09-Out	3.5	1.8	29.1	197.2	226.2	191.8	193.2
FL12	83	54.8	25-Out	4.5	1.3	26.8	101.7	128.5	179.4	111.1
FL13	31	47.0	13-Out	4.0	1.4	24.1	149.2	173.2	205.7	147.5
FL14	73	46.2	31-Out	3.8	1.4	30.7	108.5	139.1	182.8	116.7
FL15	48	48.2	14-Out	3.3	1.4	21.7	156.3	177.9	209.7	141.6
FL16	19	21.4	13-Nov	3.6	1.9	62.5	169.8	232.3	215.4	201.1
FL17	34	44.0	28-Set	3.7	1.9	50.0	165.6	215.6	212.7	187.0
FL18	126	56.6	23-Out	4.6	1.5	27.7	148.1	175.7	186.0	153.5
FL19	37	41.0	23-Set	3.5	1.9	44.9	181.8	226.7	237.8	172.3
FL20	36	39.6	03-Out	3.3	1.3	39.0	191.9	230.9	223.6	178.6
FL21	77	52.1	28-Set	4.4	1.5	53.5	208.2	261.7	234.0	197.0
FL22	52	41.9	30-Set	3.7	1.6	33.4	183.2	216.5	209.8	179.4
FL23	84	51.7	20-Out	4.4	1.4	36.2	185.8	221.9	220.7	178.4
FL24	96	48.2	12-Out	4.1	1.5	35.5	209.1	244.6	227.3	179.6
FL25	30	45.3	26-Out	3.7	1.7	21.9	155.3	177.2	192.9	147.5
FL26	34	57.2	25-Out	4.2	1.4	32.9	191.3	224.2	215.2	180.9
FL27	144	45.4	04-Out	3.9	1.4	21.9	118.9	140.7	185.2	124.5
FL28	55	39.0	06-Out	3.3	1.4	53.8	209.5	263.3	216.4	196.2
FL29	58	52.3	27-Out	3.9	1.5	13.0	166.3	179.2	202.0	147.5
FL30	105	50.8	31-Out	3.8	1.3	13.6	153.6	167.2	174.9	147.3
FL31	63	41.0	12-Out	3.5	1.6	33.4	135.8	169.2	206.9	139.9
FL32	81	67.6	09-Nov	5.6	1.5	30.9	85.8	116.7	144.7	112.4
FL33	32	45.6	28-Set	3.8	1.8	35.6	141.5	177.1	223.3	135.1
FL34	25	17.5	27-Nov	2.8	1.4	44.1	146.0	190.1	207.9	162.5
FL35	79	36.4	20-Out	3.1	1.5	21.5	147.3	168.8	213.9	134.8
FL36	**158**	48.8	17-Out	4.2	1.4	27.8	80.5	108.3	197.4	92.3
Mean	70	46.9	06-Nov	3.9	1.5	33.6	154.3	187.8	205.7	153.0

Appendix 3 *GenBank accession number DQ238053 - the o*GH *gene inter-copy region*

1: DQ238053. Reports Ovis aries growth...[gi:79013030]

```
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DEFINITION  Ovis aries growth hormone (GH) gene, GH2 allele copy GH2-N, exon 5
            and partial cds; and growth hormone (GH2) gene, GH2 allele copy
            GH2-Z exons 1, 2, 3 and partial cds.
ACCESSION   DQ238053
VERSION     DQ238053.1  GI:79013030
KEYWORDS    .
SOURCE      Ovis aries (sheep)
  ORGANISM  Ovis aries
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;
            Pecora; Bovidae; Caprinae; Ovis.
REFERENCE   1  (bases 1 to 4527)
  AUTHORS   Marques,M.R., Santos,I.C., Carolino,N., Renaville,R. and
            Cravador,A.
  TITLE     Effects of genetic polymorphisms at the growth hormone gene on milk
            yield and composition in Serra da Estrela sheep
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4527)
  AUTHORS   Marques,M.R., Santos,I.C., Belo,C.C. and Cravador,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-OCT-2005) FERN, University of Algarve, FERN - Campus
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      2761 cagttcagtt gctcagtcat gtctgactct ttgcgacccc atgaatcgca gtgtaccagg
      2821 cctccctgtc catcgccaac tcccgcagtt cactcagact cacgtccatc aagtcagtga
      2881 tgccatctag ccatctcatc ctctgttgtc cccttctcct cctgccccca atccctccca
      2941 gcatcagagt cttttccaat gagtcaaccc tttacatgag gtggccaaag tactggaatt
      3001 tcagctttag catcattcct tccaaagaaa tcccagggct gatctccttc agaatggatt
      3061 ggttggatct cctggcagtc ccagggactc tcgagagtct tctccaacac cgcagttcaa
      3121 acgcatcaat tcttcggcgt tcagccttct tcacagtcca gctctcagac tccgtacatg
      3181 accacagtag tagacatatt tgtcatctga attaaattca cccattccag tccgtttttc
      3241 tctgatatct gtgttgatgt caatgttcac tgttgccatc tcctgtttga ccacttccaa
      3301 tttaccttga ttcatggacc taacattcca ggttcctatg caatattgtt ctttacagcg
      3361 tcagacttta cttccatcac caatcgtatc tataagtggg taccattttc actttggcta
      3421 cacctcttcc tgctttctgg agttatttct ccactgagct cctgtagcat attgggcacc
      3481 taccgacctg gggagttcat tgttcagtgt catatctttt tgaattttca tactgttcat
      3541 gggattctca aggcaagaat acagaagtgg tttaccattg ccttctccag tggaccacgt
      3601 tttgtcagaa ctctcctcca tgactcatcg gtcttggatg gccctacaca gcacgggtca
      3661 tagtttcact gagttagaca agggcccagc agggaccaat tccaggatcc caggacccag
      3721 ttcaccagat gactcagggt cctgctgaca gctcaccagc tatgatggct gcaggtaagc
      3781 tcacgaaaat ccctccatt agcgtgtcct aaggggggtga tgcgggggggc cctgccgatg
      3841 gatgtgtcca cagctttggg ttttagggtt tccgaatgcg aacataggta tctacaccca
      3901 gacatttggc ccaagtttga aatgttctca gtccctggag ggaagggcag gcggggctgg
      3961 caggagatca ggcgtctagc tctctgggcc cctccgtcgc ggccctcctg gtctctccct
      4021 aggccccccgg acctccctgc tcctggcttt caccctgctc tgcctgctct ggactcaggt
      4081 ggtgggcgcc ttcccagcca tgtccttgtc ccgcctgttt gccaacgctg tgctccgggc
      4141 tcagcacctg catcaactgg ctgctgacac cttcaaagag tttgtaagct ccccagagat
      4201 gtgtcccaga ggtggggagg caggaaggga tgaatccgca accctccaca caatgggagg
      4261 gaactgagga cctcagtggt attttatcca agtaaggatg tggtcagggg agtagaaatg
      4321 ggggtgtgtg gggtggggag ggttccgaat aaggcagtga ggggaaccac gcaccagctt
      4381 agacctaggt gggtgtgttc tccccccagg agcgcaccta catcccggag ggacagagat
      4441 actccatcca gaacacccag gttgccttct gcttctctga aaccatccca gcccccacga
      4501 gcaagaatga ggcccagcag aaatcag
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*Appendix 4 Putative transcription factors binding sites at the 5' region of o*GH *gene*

Binding Factor	Position	Strand	Copy			Sequence (Search Pattern)	Score	Identifier
			GH1	*GH2-N*	*GH2-Z*			
T3R	-880	(-)			□	AGGGAG	100	HS$CS1_04
STAT[a]	-766	(-)			■	CTGGGA	100	RAT$AMGL_02
POU1F1a	-730	(-)	Δ	Δ		ATGAATA	100	HS$PL_05
AP-2[b]**, NF-1**	-720	(+)			O	TGGCC	100	HS$GRH_01
AP-2[b]**, NF-1**	-686	(+)	O	O		TGGCC	100	HS$GRH_01
STAT[a]	-671	(-)			■	CTGGGA	100	RAT$AMGL_02
STAT[a]	-640	(+)	■	■		CTGGGA	100	RAT$AMGL_02
STAT[a]	-623	(-)			■	CTGGGA	100	RAT$AMGL_02
T3R	-529	(+)	♦	♦		AGGGAG	100	HS$CS1_04
T3R-alpha	-498	(-)	♦	♦		CTTGGG	100	RAT$GRH_18
POU1F1a	-496	(-)			Δ	AATTCAG	100	HS$PL_04
POU1F1a	-490	(+)			Δ	TAAAT	100	HS$GRH_03
MAPF2, YY1	-436	(-)	x	x		GGAGC	100	RAT$MLC_04
GR	-429	(+)	✦	✦		TGTCCT	100	HS$GRH_07
T3R-alpha	-425	(+)	♦	♦		CTTGGG	100	RAT$GRH_18
POU1F1a	-403	(-)			Δ	TAAAT	100	HS$GRH_03
POU1F1a	-366	(-)			Δ	TTGCATA	100	HS$PL_01
T3R	-363	(-)	♦	♦		GGGTCA	100	HS$CS1_04
YY1	-334	(+)	x	x		CATTT	100	HS$GMCSF_03
STAT[a]	-301	(+)	■	■		CTGGGA	100	RAT$AMGL_02
delta factor, STAT5	-292	(+)	■	■		CCATTT	100	RAT$BCAS_09
YY1	-291	(+)	x	x		CATTT	100	HS$GMCSF_03
MAPF2, YY1	-245	(-)			x	GGAGC	100	RAT$MLC_04
POU1F1a	-178	(-)			Δ	ATGAAAA	100	HS$PL_10
T3R	-165	(-)	♦	♦		GGGTCA	100	HS$CS1_04
POU1F1a	-120	(+)	Δ	Δ		TAAATTATCCAT	100	RATGRH_16, HSGRH_03
POU1F1[c]	-116	(+)	Δ	Δ		TTATCCAT	100	HS$GRH_05
STAT5B	-101	(-)			■	TTCTGAGAA	88.13	HS$P21WAF1_10
Zn-15	-99	(+)	▼	▼		CTGTCAGTGG	89.44	RAT$GRH_24
POU1F1a	-86	(+)	Δ	Δ		TTGCATA	100	HS$PL_01
AP-1	-83	(+)			✖	ATGAATCATC	89.44	HS$PL_06
POU1F1a	-81	(+)	Δ	Δ		TAAAT	100	HS$GRH_03
YY1	-80	(-)	x	x		CATTT	100	HS$GMCSF_03
AP-2[b]**, NF-1**	-64	(-)			O	GGCCA	100	HS$GRH_01
T3R	-51	(+)	♦	♦		AGGGAG	100	HS$CS1_04
T3R	-48	(+)			♦	GGGTCA	100	HS$CS1_04

[a] - IL-6 RE-BP, STAT1, STAT5A, STAT5B, STAT6; [b] - AP-2, AP-2alphaA, AP-2alphaB; [c] - POU1F1, POU1F1a, POU1F1b, POU1F1c

Δ - POU1F1, POU1F1a, POU1F1b and/or POU1F1c (pituitary-specific transcription factor 1); ✖ - Ap-1 (activator protein 1); O - AP-2 and NF-1, AP-2alphaA and AP-2alphaB (activator protein 2) and nuclear factor 1; ✦ - GR (glucocorticoid receptor); ♦ - T3R and/or T3R-alpha (3,5,3'-triiodothyronine receptor); ■ - STAT1, STAT5A, STAT5B and/or STAT6 (signal transducer and activator of transcription); ▼ - Zn-15 (zinc finger protein); **x -** YY1 (yin and yang factor 1).