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Isolation and molecular characterization of the fatty acid synthase gene from an obese pig breed

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"*Success consists of going from failure to failure without loss of enthusiasm.*"

Winston Churchill

"*You must be the change you wish to see in the world*."

Mahatma Gandhi

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ABBREVIATION LIST

7TM – Seven transmembrane receptors

ACC – Acetyl CoA carboxylase

ACP – Acyl carrier protein

AFW – Abdominal fat weight

AL – Alentejano

AMP – Adenosine monophosphate

AMPK – AMP-activated protein kinase

ATP – Adenosine triphosphate

BFT – Backfat thickness

BMI – Body mass index

bp – Base pairs

BSA – Bovine serum albumin

BW – Body weight

C – Carbon

ºC – Celsius degree

ca. – circa

cAMP – Cyclic adenosine monophosphate

cds – Coding sequence

cDNA – Complementary deoxyribonucleic acid

CiC – Citrate carrier

CO_2 – Carbon dioxide

CoA – Coenzyme A

CVD – Cardiovascular disease

DH – Dehydratase

DMSO – Dimethyl sulfoxide

dNTPs – Deoxynucleotide Triphosphates

DNA – Deoxyribonucleic acid

EC – Enzyme classification number

E. coli – Escherichia coli

EDTA – Ethylenediaminetetraacetic acid

e.g. – exempli gratia

EGCG – Epigallocatechine-3-gallate

ER – Enoyl reductase

FA – Fatty acid

FAD – Flavin adenine dinucleotide

$FADH_2$ – Reduced form of flavin adenine dinucleotide

FAS – Fatty acid synthase

FASN – Fatty acid synthase gene

FFA – Free fatty acid

FW – Forward

g – Grams

g – Gravitational acceleration

h – Hour

H_2O – Water

HDL – High-density lipoprotein

kD – KiloDalton

KS – Ketoacyl synthase

KR – Ketoacyl reductase

IKKβ – IκB kinase-β

IL – Interleukin

IMF – Intramuscular fat

IPTG – Isopropyl-[beta]-D-thiogalactopyranoside

IRS – Insulin receptor substrate

IUBMB – International Union of Biochemistry and Molecular Biology

IUPAC – International Union of Pure and Applied Chemistry

JNK – Jun kinase

Kb – Kilobase

kDa – Kilodalton

kJ – Kilojoule

LDL – Low-density lipoprotein

LEPR – Leptin receptor gene

LPS – Lipopolysaccharide

M – Molar

MAT – Malonyl-acetyl transferase

MCP – Monocyte chemoattractant protein

min – Minute

mg – Milligrams

MJ – Megajoule

mL – Milliliters

mM – Millimolar

M-MLV – Moloney murineleukemia virus

mRNA – Messenger RNA

MUFA – Monounsaturated fatty acid

NAD^+ – Oxidized form of nicotinamide adenine dinucleotide

NADH – Reduced form of nicotinamide adenine dinucleotide

NADPH - Reduced form of nicotinamide adenine dinucleotide phosphate

NCBI – National Center for Biotechnology Information

nm – Nanometer

nsSNP – Non-synonymous single nucleotide polymorphism

O_2 – Molecular oxygen

P – Pig

PCR – Polymerase chain reaction

PKC – Protein Kinase C

PUFA – Polyunsaturated fatty acid

QTL – Quantitative trait locus

rpm – Revolutions per minute

RNA – Ribonucleic acid

RNase – Ribonuclease

RT – Reverse transcription

RT-qPCR – Reverse transcription followed by quantitative polymerase chain reaction

RV – Reverse

s – Second

SCD – Stearoyl CoA desaturase

SFA – Saturated fatty acid

SNP – Single nucleotide polymorphism

SOCS – Suppressor of cytokine signaling proteins

SREBP1-c – Sterol regulatory element binding protein 1-c

SUB – Subcutaneous adipose tissue

TBE – Tris-Borate-EDTA

TE – Thioesterase

TLR – Toll-like receptor

TNF – Tumor necrosis factor

Tris – Tris (hydroxymethyl) aminomethane

U – Unit

UFA – Unsaturated fatty acid

UV – Ultraviolet

V – Volt

w/v – weight/volume

X-Gal – 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

µL – Microliters

Nucleotide bases

A	Adenine
T	Thymine
G	Guanine
C	Cytosine

Amino acid nomenclature follows International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry and Molecular Biology (IUBMB) recommendations (1984)

A	Alanine
C	Cysteine
D	Aspartate
E	Glutamate
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine

M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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RESUMO

Isolamento e caraterização molecular do gene *fatty acid synthase* num porco obeso

O porco Alentejano é uma raça autóctone do sul de Portugal geneticamente idêntica ao porco Ibérico. Esta raça obesa é caraterizada por um crescimento lento e uma capacidade adipogénica precoce e elevada. A formação do tecido adiposo em suínos é obtida em 80% via síntese *de novo*, apesar de também poder ocorrer síntese independente de ácidos gordos intramusculares. O porco Alentejano apresenta a característica *genótipo "poupador"* (*thrifty genotype*), responsável pelo elevado potencial e maior capacidade de deposição de tecido adiposo que outras raças comerciais magras.

O objetivo deste estudo foi determinar a sequência genética do enzima lipogénico *fatty acid synthase* (FAS) do tecido adiposo subcutâneo de um porco obeso de raça Alentejana. De acordo com a pesquisa feita, esta é a primeira publicação de uma sequência parcial deste gene em porcos Alentejanos. Através da clonagem de produtos de PCR, foram detetados três polimorfismos: c.6361C>T, c.6632C>A e c.6693C>T., sendo que os últimos dois conduzem a alterações no resíduo traduzido.

Palavras chave: Suíno; raça Alentejana; fatty acid synthase; ácidos gordos; lipogénese; deposição; adipócito; síntese *de novo*; gordura intramuscular; sequenciação.

ABSTRACT

The Alentejano (AL) pig is an autochthonous breed genetically similar to the Iberian pig. This obese breed is characterized by slow growth rates and precociously high lipogenic activity. *De novo* synthesis is responsible for about 80% of all fatty acid (FA) synthesis in swine adipose tissue, although independent intramuscular FA synthesis can also occur. The AL breed features the *thrifty genotype*, responsible for a higher potential and ability for fat deposition than other industrial lean breeds.

The aim of this study is to determine the gene sequence of the porcine fatty acid synthase (FAS) lipogenic enzyme of an obese AL pig in subcutaneous adipose tissue. To our knowledge this is the first publication of a partial sequence of this gene in AL pigs. Through cloning of polymerase chain reaction (PCR) assayed products three polymorphisms were detected: c.6361C>T, c.6632C>A and c.6693C>T, the latter two change the translated residue.

Key Words: Swine; Alentejano breed; lipogenesis; deposition; adipocyte; *de novo* synthesis; intramuscular fat; Fatty acid synthase; sequencing

I. INTRODUCTION

Sus scrofa domesticus, commonly known as domestic pig, is a eutherian mammal that has shared a natural bond with humans since the beginning of its domestication all across Eurasia around 10000 years ago up to modern breeding practices and biomedical models (Groenen et al., 2012).

Pigs are commonly raised for meat, which is the most important animal protein food source, and feed a majority of the global population. As an important meat source in a world with increasing and demanding food needs, particularly due to developing countries and exponential human population growth, this particular livestock species has increased its global population by 13 percent in the last two decades (FAO, 2013).

As a component of meat, fat, particularly saturated fat, is generally considered as deleterious to the health of the consumers, and is associated with higher chances on developing heart disease. Therefore animal feed strategies and processing technologies are nowadays increasingly used to alter meat composition to be more consistent with human dietary guidelines. Nevertheless, these strategies and techniques need to be based on the knowledge of the genetic potential and physiological characteristics of the animals to be efficient.

The perception of the adipose tissue being solely an energy storage organ has been extended to include an endocrine organ that plays a pivotal role in relation to energy homeostasis and metabolism. Apart from secreting free fatty acids, adipose tissue produces and releases numerous proteins and substances with autocrine, paracrine and endocrine functions.

The Alentejano (AL) pig, *Sus mediterraneus*, is an autochthonous breed, reared in the southern area of Portugal. This breed has been scarcely selected through centuries and due to its trend to fat deposition, is smaller in size and presents poorer meat yields than modern commercial breeds. This precocious and high trend of fat storage has been found not only in AL and Iberian pigs, but also in other animal species and even in humans, being named as “thrifty” genotype. In fact, the thrifty genotype is an adaptive mechanism to the environment allowing accommodation to cycles of feasting and famine (Neel, 1962) One intrinsic mechanism of the thrifty genotype has been recently associated in humans with a syndrome known as leptin resistance. Leptin resistance has been identified with disruptions of signal transduction processes at the level of leptin receptors with effects on food behavior and obesity (Lubis et al., 2008). Thus, leptin resistant individuals are obese genotypes with elevated leptin levels but unable to suppress feeding when food is in excess (Myers et al., 2008). In the Iberian pig a leptin receptor gene polymorphism has also been detected, with effects on food intake, body weight and fat deposition (Munoz et al., 2009; Ovilo et al., 2005).

Porcine genome sequencing it's of most importance because it provides a valuable feature for this livestock species progress, while enables continuous studying for multiple disease-causing variations extending the swine potential as a biomedical model. (Groenen et al., 2012)

This study's main goal is to sequence a lipogenic enzyme gene, fatty acid synthase (FAS), of an obese AL pig. Genotyping was performed in the most important tissue involved in the lipid metabolism, subcutaneous adipose tissue.

II. LITERATURE REVIEW

1. The Alentejano pig - Historical and Local Background

1.1. The Swine

The autochthonous pig breed Alentejano (AL) or *Black* pig is a traditionally raised breed that produces high quality meat products thanks to its particular production system and very particular genotypic features (Freitas et al., 2007; Lopez-Bote, 1998).

AL breed is reared in the southern region of Portugal and descends from *Sus scrofa mediterraneus* lineages, just as the genetically similar Iberian pig. Crosses between these two *Sus mediterraneus* descendants are common throughout the years. The Iberian pig term is accepted and used among many authors, to refer the several breeds existing in the Iberian Peninsula region (Martins et al., 2012; Nunes, 1993).

The Iberian Peninsula constitutes a strong historical and geographical unit that throughout history contributed to the formation of many local animal breeds, including the AL/Iberian pig. Nevertheless, there still is considerable genetic differentiation and relatively high genetic diversity found in Iberian breeds, most of them considered genetic resources (Canon et al., 2011; Matos, 2000; Nunes, 1993).

This Iberian swine branch presents, generally, several features: dark skin and hair color (if present); pointed snout; short and strong limbs. These morphological traits reflect the adaptation of the swine to the local conditions, particularly in terms of resistance to sunstroke and high temperatures (Fabuel et al., 2004; Nunes, 1993). Reproductive and zootechnical parameters are presented in several works (e.g. Freitas et al. (2006)).

Swine production can be divided into three distinct types depending on the final product: meat (*e.g.* Duroc, German Landrace, Pietrain); bacon (*e.g.* Danish Landrace, Tamworth) and fat (*e.g.* AL/Iberian, Gascon, Casertana). Meat-type pig breeds are characterized by high daily weight gains and are slaughtered with a low body fat content and constitute the main source of pork in developed countries, while fat-types have a lower BW gain and produce carcasses rich in fatness (Freitas et al., 2007; Switonski et al., 2010).

The AL is of smaller size and fatter, besides holding higher intramuscular fat (IMF) content than other Iberian breeds. This obese breed is characterized by slow growth rates

and precociously high lipogenic activity. Traditionally, these pigs are fed until they reach a body weight (BW) between 150 and 170 kg and 18-24 months of age, when they are ready for slaughter (Lopez-Bote, 1998; Martins et al., 2012; Nunes, 1993).

Unlike modern lean-type breeds, the AL is a local raised unselected breed with considerable genetic differentiation and poor productive traits. Nevertheless, until the second half of the 20th century, the AL pig breed was, economically, the most important Portuguese pig breed. Their meat, rich in IMF, is mainly used for the manufacture of high-quality dry-cured products. More recently, an increased interest on these products and in fresh meat from Al pigs has been observed among consumers, sustained by their intrinsic quality and healthiness (Freitas et al., 2007; Freitas et al., 2006; Gonzalez-Anover et al., 2010; Teixeira and Rodrigues, 2013) (see below).

Swine fatty acid (FA) synthesis is mainly through *de novo* synthesis (around 80% or more on adipogenic breeds) in adipose tissue with the possibility of independent intramuscular FA synthesis (Hood and Allen, 1973; Martins et al., 2012). Besides a higher content of fat, carcasses of AL and Iberian pigs generally present a higher content of oleic acid and (Estévez et al., 2006; Martins et al., 2012), consequently, a lower content of saturated fatty acids (SFA) commonly associated with the development of cardiovascular diseases (CVDs) (Estévez et al., 2006).

The production of AL and Iberian pigs is deeply rooted to the Mediterranean ecosystem. The contribution of this swine breed to the preservation and sustainability of its ecosystem is not common among the swine production industry (Lopez-Bote, 1998; Matos, 2000; Pugliese and Sirtori, 2012). In fact, local pig breeds and their production systems have been able to respond to the high criteria and expectations of modern society in regard to environment, animal welfare, and food quality and healthiness (Pugliese et al., 2013).

1.2. Production System - The Influence of *Montado* and *Montanheira*

Alentejo, the southern region of Portugal where AL pig is reared, is full of extensive plains of poor, substantially degraded soils where livestock production represents an important local economic activity. The agro-silvo-pastoral system characteristic of this region is based on the *montado*. (Freitas et al., 2006).

The feed availability offered by the Portuguese *montado* is seasonal and also varies from year to year (Lopez-Bote, 1998; Nunes, 1993).

Traditionally, a diverse diet is supplied to pigs in extensive free-range conditions in long production cycles. Fattening periods during late October until February take full advantage of the *montado* landscape opposing the periods of scarcity where feed supplementation based on cereals/commercial diets might be required. The fattening

montanheira period explores local natural resources such as native pastures and *Quercus rotundifolia* and *Quercus suber* fruits (acorns), and is perfectly adapted to the AL extensive production. Acorns are low in protein, rich in the monounsaturated fatty acid (MUFA) oleate (C18:1) and pastures on polyunsaturated fatty acids (PUFA) linoleic (C18:2) and linolenic (C18:3) acids, besides fiber, protein and antioxidants such as alpha-tocopherol. Native pasture consumption also contributes for the detoxification due to the tannin content of acorns, while complementing the acorns low-protein content. In the traditional production systems, pigs are finished in the *montanheira*, where they are submitted to a quick and intense fattening period (Gonzalez-Anover et al., 2010; Matos, 2000; Nunes, 1993).

Modern explorations maintained the most dominant and interesting traits of the traditional production system, particularly, fattening pigs in *montanheira* associated with the exploration of reproductive sows. Nevertheless, there isn't a uniform production system since mating season, feed management, weight and age of slaughter varies among farms (Freitas et al., 2006).

More recently, an evolution in this production system has been verified, with the objective of a more intensified production. The feeding of pigs for example, is based on commercial MUFA-enriched diets having alpha-tocopherol supplementation up to 200 mg/kg in order to simulate the effects of the *montanheira* in the traditional free range feeding system (Daza et al., 2005). The growing interest on the semi-extensive camping system for swine management (requiring fewer workers) is another example (Matos, 2000).

Nowadays, pigs used to produce hams and dry-cured products are slaughtered at 150-170 kg BW and 14 to 24 months of age and are finished in *montanheira* (high quality products) or semi-extensive production systems (lower quality products). When sausages are the final product, pigs are slaughtered at 120-140 kg, and 14-20 months of age. Finally, for fresh meat, pigs are slaughtered at 90-100 kg and 8-14 months of age. These two final products are generally obtained from pigs reared in the semi-extensive and intensive production systems (Freitas et al., 2006).

Pork is one of the most produced and consumed meat-types in the world, despite the negative assumptions broadly made. The widespread opinion about the unhealthy effects of this meat is mainly related to its SFA and cholesterol content (FAO Food and Agricultural Organization of the United Nations., 2013a; Teixeira and Rodrigues, 2013; Wood et al., 2008).

Commercial lean-type pig breeds, developed to reduce pork total lipid content, have better productive traits when compared to AL and other unselected breeds. They are raised on intensive systems, using advanced technology which translates into an improvement in terms of carcass meat yield and manufacturing of low cost products. Nevertheless, animal products raised traditionally foster now more interest among consumers. This current interest might be associated with the characteristics of the extensive production systems that guarantee quality to the final product, increasing

consumer interest in organic and natural pig production. The high IMF content in AL/Iberian pork products is responsible for much appreciated organoleptic traits such as tenderness, juiciness and flavor (Gonzalez-Anover et al., 2010; Pugliese et al., 2013; Switonski et al., 2010).

AL and Iberian products represent an important part of traditional pork production and have significant commercial value. Furthermore, the distinctive FA profile of its meat, rich in unsaturated fatty acids (UFAs) and lower SFA content, contributes to an improved consumer approval of these products.

2. Swine-based research

2.1. Swine as Human Biomedical Model

The use of animal models for research of human diseases is a general practice that provides important information for development of prevention strategies and appropriate treatments. Better models rely on anatomic and physiological similarities to enable reliable extrapolations to humans.

Rodent subjects are widely adopted for human health studies. Nevertheless, rodent models occasionally fail to fully mimic human conditions, so an increasing need for animals closer to humans to further study diseases such as cystic fibrosis, diabetes or obesity, among others, is required (Cirera et al., 2014; Spurlock and Gabler, 2008; Walters et al., 2012).

In the last three decades, the adoption of pigs as human biomedical research model has broadly increased, particularly for general surgical models of most organs and systems, for cardiovascular research and in digestive system models, and more recently in transplantation and xenographic research through genetic engineering (Hamamdzic and Wilensky, 2013; Walters et al., 2012; Whyte and Prather, 2011).

Completion of the swine genome is currently simplifying and broadcasting the use of swine models in several research areas. For instance, it is now possible to search for single nucleotide polymorphisms (SNPs) of specific human disease genes and compare them to the ones existing in pigs. Through modern genomic modification techniques, swine gene functions can be altered, aiding the development of novel disease models based on transgenes on areas as diverse as cardiovascular disease, xenotransplantation and neurodegenerative diseases (Walters et al., 2012; Whyte and Prather, 2011).

The vast conserved homology saved by both, swine and human, genomes is one of the main features that support the extensive use of swine as biomedical models. Both species are omnivorous and their organs share similar functional features. Furthermore, resemblances in size, anatomy and physiology make the swine a suitable model for research in gene and cell therapy, xenograft and allograft procedures and other fields of regenerative medicine. Similar disease progression has allowed the possibility of a broad range of distinct biomedical swine research models. All-season breeding and large litter sizes provides great availability of individuals that hold short generation intervals (12 months) and gestation lengths (114 days), plus an early sexual maturity (5-8 months) and short lifespan (10-20 years). Handling pigs from the same litter, or cloned or transgenic pigs, eases genetic mapping. Choosing the right breed and age allows the execution of various surgical and non-surgical procedures generally used in human medicine, including catheterization, heart surgery, valve manipulation, endoscopy and broncho-alveolar lavages. In opposition, some of these procedures are found hard to practice in other animal models including rodents (Lunney, 2007; Meurens et al., 2012; Rothschild and Ruvinsky, 2011).

Swine also allow the possibility to perform time studies, image internal vessels and organs, and collect repeated peripheral samples and, at kill, detailed tissue samples. Access to well defined cell lines from different tissues eases gene expression and drug susceptibility testing, for example. Moreover, swine full genomic code is already available and its high sequence and chromosome structure homology with humans will provide a further improvement to its genomic and proteomic tools. Swine models are inexpensive and ethically more acceptable than primates and other animals since they are used for human feeding as well. Table 1 summarizes the general advantages of swine biomedical models (Lunney, 2007; Meurens et al., 2012).

Table 1. Major advantages of a swine biomedical model.

Adapted from Lunney (2007) and Meurens et al. (2012)

- Human sized - particularly miniature pigs
- Similar anatomy and physiology to humans
- Availability of individuals
- Advanced cloning and transgenic technology
- Availability of numerous well defined cell lines
- Similar disease progression (metabolic, infectious and across species diseases)
- Ability to perform various surgical procedures and of collecting multiple samples by choice
- Complete swine genome sequence availability with improving genomic and proteomic tools
- High sequence and chromosome structure homology with humans

2.2. Swine Biomedical Models for Obesity and type-2 Diabetes Studies – Fatness Genetics

Obesity is a chronic disease characterized by excessive body fat in tissues due to over accumulation of fat stores at the adipocyte level. In Humans, obesity is effectively diagnosed when body mass index (BMI) is superior to 30 kg m^{-2}. Obesity is a major health risk factor for a number of chronic diseases, including diabetes, cardiovascular diseases, and cancer. Among adults, worldwide prevalence of obesity is over 10% and prevalence of obesity and overweight ascend up to more than 30%. In Europe and Northern America these results reach 60 and 70% of the adult population, respectively. Despite these alarming results, trends are expected to climb even more in coming years (FAO Food and Agricultural Organization of the United Nations., 2013b; Spurlock and Gabler, 2008).

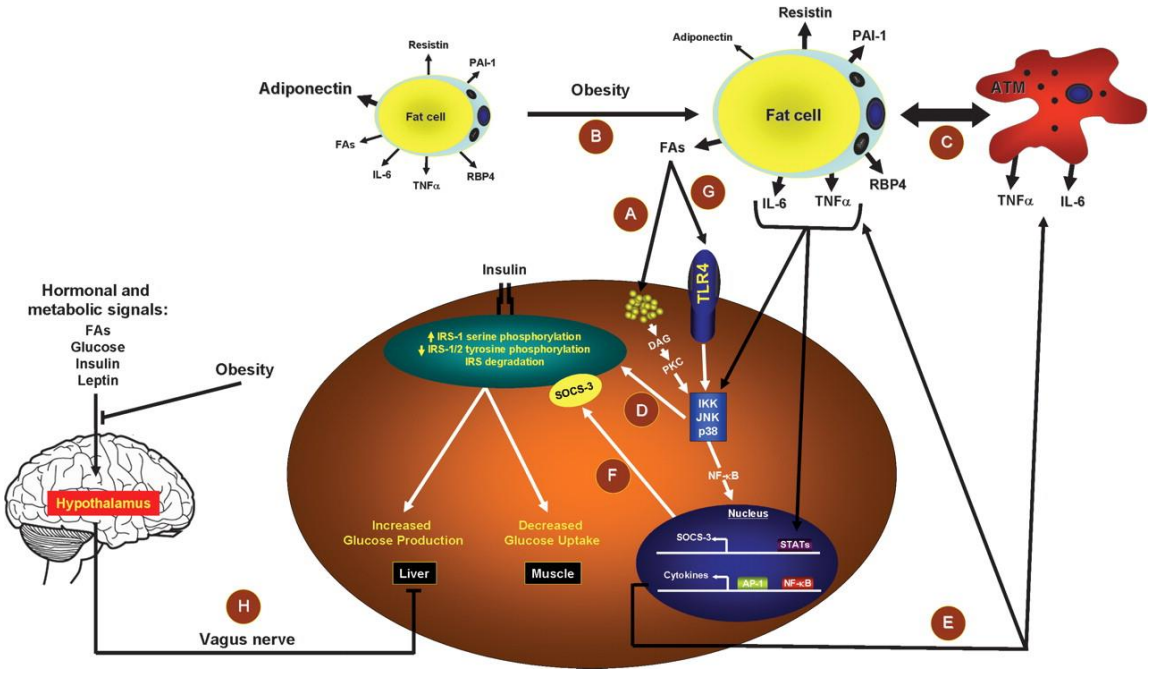
Biochemical mechanisms responsible for obesity haven't yet been fully identified and while rodent models have been extensively experimented for obesity research, their translational utility is less effective than pigs. This fact seems to be related with some differences at metabolic and physiological levels between rodents and humans. For example, several adipokines, such as adipsin or resistin, cytokines secreted by the adipose tissue, appear to have different activities (Arner, 2005; Spurlock and Gabler, 2008). Furthermore, swine, as humans, are devoided of brown adipose tissue postnatally. This resemblance is very important since brown adipose tissue can induce heat production and therefore regulate energy balance (Harms and Seale, 2013; Spurlock and Gabler, 2008).

Several studies suggest that obesity is highly correlated with macrophage accumulation in adipose tissue. Activated macrophages secrete various cytokines and chemokines involved in systemic inflammation such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and monocyte chemoattractant protein (MCP)-1, the later contributing to infiltration of adipose tissue with immunocytes (Harford et al., 2011; Spurlock and Gabler, 2008; Weisberg et al., 2003; Xu et al., 2003).

As obesity, type-2 diabetes is a major cardiovascular risk factor, namely for developing coronary heart disease (Haffner et al., 1998).

Chronic inflammation induced by obesity can develop insulin resistance. It is widely known that high plasma FA concentrations are closely related to obesity because expansion of the adipose tissue entails FA release from the adipocyte depots. Furthermore, obesity-associated changes induce alterations in the secretion patterns of adipokines that modulate insulin signaling (see illustration 1, path B). Recent in vitro studies have demonstrated that SFAs stimulate tissue inflammation the same way as bacterial lipopolysaccharides (LPS), through activation of toll-like receptors (TLR)-4 (illustration 1, path G) which indirectly promote cell inflammation with further production of pro-inflammatory TNF-α and IL-6 through the activation of protein kinases such as Protein Kinase C (PKC, EC 2.7.11.13), IκB kinase-β (IKKβ, EC 2.7.11.10), Jun kinase (JNK, EC 2.7.11.24) and the inhibitor of nuclear factor-κB (NF-κB, EC 2.7.11.10) (illustration 1,

path E). These kinases are responsible for the serine phosphorylation of insulin receptor substrates (IRS) that inhibits insulin signaling (illustration 1, path D). Moreover, the adipokine induced family proteins SOCS-3 conflict with IRS-1 and IRS-2 by phosphorylation or proteosomal degradation (illustration 1, path F). Main consequences to insulin sensitivity are an increased glucose synthesis in the hepatocyte, while on the myocyte the glucose uptake is decreased (Bjorntorp et al., 1969; Chait and Kim, 2010; Qatanani and Lazar, 2007; Spurlock and Gabler, 2008).



Adapted from Qatanani and Lazar (2007)

Illustration 1. Insulin resistance induced by obesity might involve a complex network of endocrine, inflammatory and neuronal pathways.

Another adipokine closely related to obesity and inflammation, in pigs as in humans, is adiponectin. Adiponectin is a protein secreted exclusively by adipose tissue that modulates lipid metabolism by inducing AMP-activated protein kinase, AMPK (EC 2.7.11.31), activity which deactivates acetyl CoA carboxylase, ACC (EC 6.4.1.2) and, consequently, suppresses carbohydrate transformation into lipid reserves by inhibiting *de novo* fatty acid synthesis. A small number of studies showed that lower concentrations of circulating adiponectin are found in obese and among insulin resistant subjects, suggesting anti-inflammatory effects. On the other hand, leptin is an adipocyte secreted hormone that regulates physiological functions in energy shortage periods and, when absent, leads to major metabolic disturbances due to increased insatiability. Adiponectin and leptin are found to be reciprocally regulated (Brennan and Mantzoros, 2006; Qatanani and Lazar, 2007; Spurlock and Gabler, 2008; Wu et al., 2003).

Anti-inflammatory effects by adiponectin have been reported in both swine and human adipose tissue. Production of pro-inflammatory cytokines as IL-6 and TNF by activated macrophages is suppressed by adiponectin. On the other hand, IL-6 and TNF

regulate adiponectin expression by inhibiting its mRNA expression (Spurlock and Gabler, 2008).

Further studies, using more swine subject models, might uncover more or confirm about the crucial role that these and other factors might have in inflammation, obesity and obesity-related diseases. These studies will include the search for gene mutations, polymorphisms and allele comparison in relevant genes, taking advantage of the recent availability of the full swine genome sequence and development of techniques such as SNP microarrays (Bruun et al., 2003; Spurlock and Gabler, 2008; Switonski et al., 2010; Wulster-Radcliffe et al., 2004; Yokota et al., 2000).

Evaluation of genetic predisposition through comparative genomic studies on obesity candidate genes and search for fatness quantitative trait loci (QTLs) are current fields of obesity research in expansion. All these obese swine traits have high polygenic background. To date, more than 100 loci have been identified and associated with obesity-related traits by genome-wide association studies (GWAS) as well as several candidate genes. Nevertheless, this approach is revealing to be rather limited since the individual expression value of current identified candidate genes is quite low when explaining fatness phenotypic traits. This is thought to be related with the high polygenic background involved in the determination of fatness traits, as well as the possibility of epistatic effects between potential genes (Cirera et al., 2014; Schook et al., 2005; Switonski et al., 2010).

Modern pig breeding has also found interest in studying swine genetic background in order to improve livestock production. Most important and studied swine fatness traits are backfat thickness (BFT), abdominal fat weight (AFW), IMF content and FA composition. BFT and AFW are related to fattening efficiency while IMF and FA composition affect quality meat traits and display nutritional value. IMF levels vary between breeds and muscle type. In *Longissimus dorsi*, for example, one of the most valuable muscles, the average IMF content in selected breeds is about 1.7%, while in AL/Iberian pigs this is much higher and may attain 6%. Furthermore, lean-type pig breeds are selected towards lower levels of BFT and growth of leaner tissues (Daza et al., 2006; Switonski et al., 2010).

Limitations occur when translating conclusions about porcine candidate genes to humans and vice-versa in fatness deposition. While studies of human obesity focus on BMI, traits studied in swine are concerned with different aspects of fatness which span candidate genes that may not prove to be as relevant for human obesity research. Nevertheless, particular animal models, such as the obese Ossabaw pig, are becoming increasingly valuable assets to understand the development of human obesity, obesity-induced insulin resistance and ultimately the metabolic syndrome and cardiovascular diseases. (Switonski et al., 2010)

2.3. Potential of the AL Breed

Several recent research studies about human obesity and development of metabolic syndrome have been done with Ossabaw pigs as models. For example, this breed can easily and efficiently simulate human atherosclerosis. This swine breed is exclusively found on Ossabaw Island (Georgia, USA) and features a thrifty genotype which drivens it to feast in excess, easily become obese and develop all pathologies clustering metabolic syndrome such as obesity with severe visceral adipose expansion, primary insulin resistance, glucose intolerance, dyslipidemia and hypertension. Presence of three out of five of these symptoms is required to characterize metabolic syndrome in humans. Ossabaw swine can become overly obese even when fed with diets with no added fat but rich in carbohydrates. Recent studies suggest that these pigs are descendent from an Iberian branch which may explain their morphological and metabolic similarities (Brisbin and Sturek, 2009; Faris et al., 2012; Hamamdzic and Wilensky, 2013; Spurlock and Gabler, 2008; Toedebusch et al., 2014).

Understanding of the pathophysiology of metabolic syndrome has been delayed by the lack of efficient human-like models. However, Ossabaw breed happens to be one of the best models for studying metabolic syndrome in humans due to the easiness in developing all its associated pathologies at once. As the Ossabaw strain, AL pigs hold high propensity for obesity, due to an adaptive thrifty genotype, including a great capacity to accumulate intramuscular and epidermal fat (Hamamdzic and Wilensky, 2013).

Iberian pigs hold a polymorphism on the leptin receptor gene (LEPR) causing a condition commonly known as *leptin resistance*, characterized by failure in feeding suppression due to high concentrations of leptin found in obese individuals. This genetic feature results in implications at food intake, BW and fat deposition patterns, promoting obesity development (Munoz et al., 2009; Ovilo et al., 2005).

Recently, a study conducted by Torres-Rovira et al. (2012) concluded that within three months, Iberian sows with access to an *ad libitum* diet enriched with saturated fat, developed all five criteria associated to metabolic syndrome. This suggests that AL/Iberian pigs could be a valuable human model for further comprehension on mechanisms involved in obesity and its associated comorbities.

3. Lipid Metabolism

This chapter will be focused in introducing mammalian lipids as important energy stores in adipose tissue, and their successive transformations, depending on the current metabolic needs.

3.1. Introduction to Lipids – The Fatty Acids

Lipids are a class of biomolecules that display a variety of distinct biological and structural functions. Lipids integrate cell membranes, carry fat soluble vitamins, and play vital roles as enzyme cofactors, hormones, and electron carriers, among others. Nevertheless, their main function lies in providing energy (Berg et al., 2012; Hussain et al., 2013; Lehninger et al., 2008).

Animals store energy mainly in the form of fat so that proteins don't have to be used, allowing them more important roles such as building and repairing tissues. In addition, storing calories as fat is more efficient than with the same mass of carbohydrates in the form of glycogen. Energy stores offer survival for long periods of food deprivation and its associated efficiency is thought to offer important advantages to animals during evolution (Lehninger et al., 2008; Semenkovich, 1997).

Animals store energy in the form of FAs, the simplest class of lipids. They contain a methyl group, a long hydrophobic hydrocarbon chain varying between 4 and 36 carbon atoms, and a terminal carboxylate group (illustration 2). FAs can be stored in excess amounts in the adipose tissue and oxidized in all tissues, particularly liver and muscle, acting as a major source of fuel to cells. In the form of phospholipids (mainly phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin), FAs form the backbone of cell membranes and contribute for their fluidity and functionality. FAs can also be found freely, circulating bound to albumin or integrated in triacylglycerols and waxes (Lehninger et al., 2008; Tvrzicka et al., 2011; Vance and Vance, 2008) (see illustration 3).

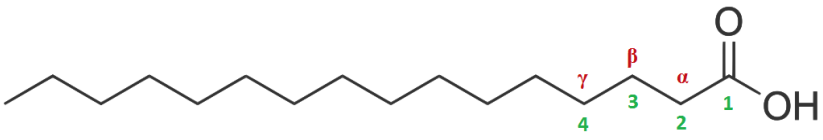


Illustration 2. The structure of palmitic acid or palmitate (C16:0), one of the most common FAs found in animals. Carbon count starts from the carboxylic group (1), the first carbon after the carboxyl carbon is called α (2) while the next is known as β (3) and the fourth as γ (4).

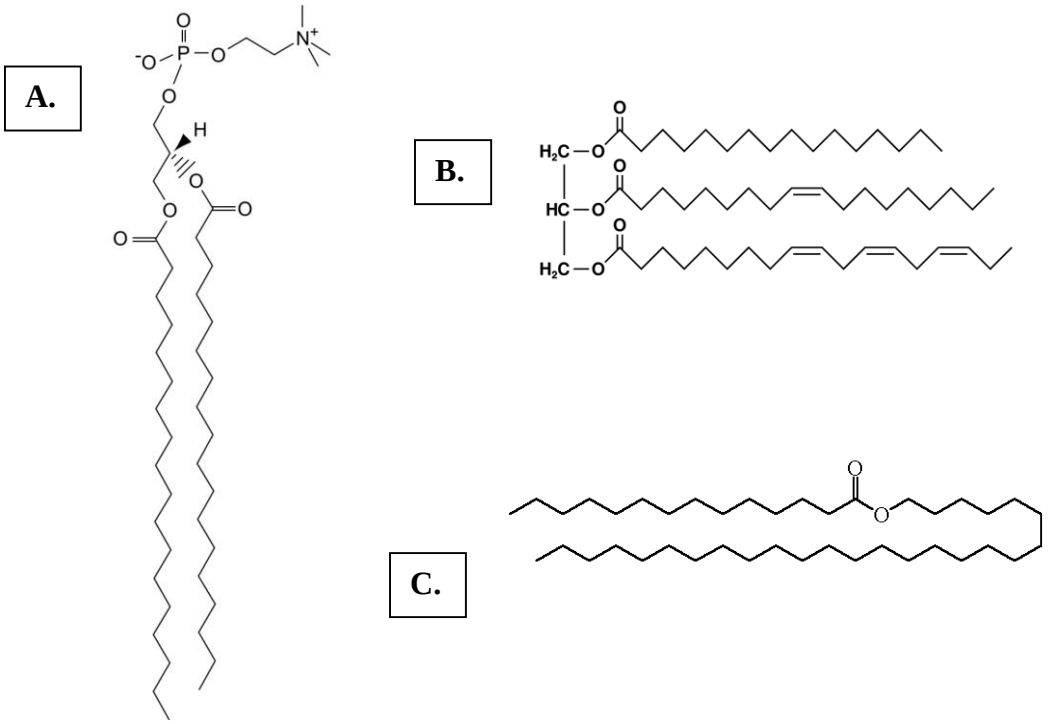


Illustration 3. Distinct types of fatty acid–containing compounds. **A.** The structure of phosphatidylcholine (glycerolphosphate-based lipid), a phospholipid based on a glycerol backbone along with a phosphate group, a choline group and two stearic acids (C18:0); **B.** The structure of a triacylglycerol comprising a glycerol and three distinct FA, from top to bottom: palmitic acid (C16:0), oleic acid (C18:1, Δ^9) and alpha-linolenic acid (C18:3, $\Delta^{9, 12, 15}$); **C.** The structure of the animal wax ceryl myristate, an ester of ceryl alcohol and myristic acid (C14:0).

Fatty acids can be defined as being saturated or unsaturated, depending on the absence or presence of double bonds between two carbon atoms in the aliphatic chain, respectively. Furthermore, UFAs display cis-trans isomerism, describing the orientation of the alkyl groups around the double bond. The trans isomer of a given FA can rarely be found in nature. Trans FAs can be obtained in dairy products or their meat and can also be produced by partial hydrogenation of fish and vegetable oils. Large dietary intakes of trans FAs are strongly correlated to increased levels of triacylglycerols and LDL (low-density lipoprotein) cholesterol and lower levels of HDL (high-density lipoprotein) on blood, conducing to a higher risk in developing cardiovascular diseases (Chilliard, 1993; Lehninger et al., 2008).

Delta (Δ) FA nomenclature is a simple mean of classification and identification of different FAs by specifying the chain length and number of double bonds, separated by a colon. In addition the number superscripted above the Δ represent the number of carbons from the carboxylic acid end to the first carbon in the double bond (Lehninger et al., 2008) (illustration 4). This simplified nomenclature, along with common names, will be recurrently used throughout this thesis.

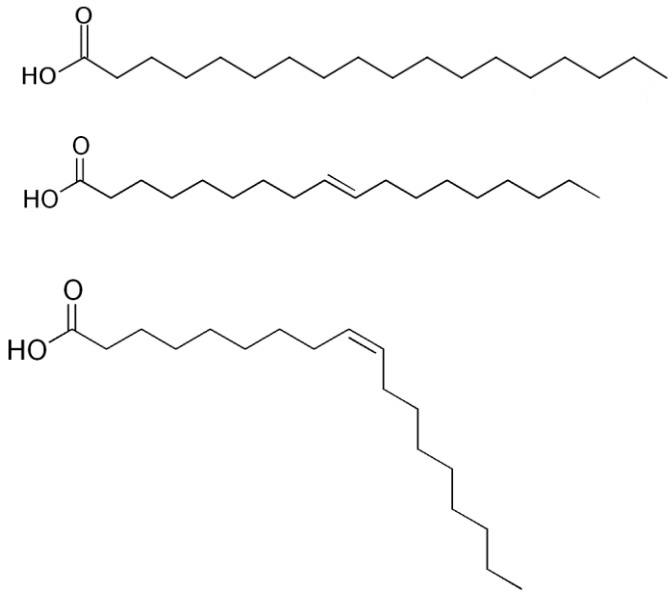


Illustration 4. Example of fatty acids classification using the delta nomenclature, from top to bottom: stearate (C18:0), trans-oleate (C18:1, Δ^9) and cis-oleate (C18:1, Δ^9).

3.2. Fatty Acid Catabolism

Animal cells can obtain FAs from three distinct pathways: fats ingested in the diet, lipolysis of lipids stored in cells and *de novo* synthesis.

Triacylglycerols are formed by esterification of the three hydroxyl groups of glycerol by carboxyl groups of organic acids. They function as highly concentrated energy stores accumulated in cell depots, mainly in liver and adipose tissue. Their energy yield from complete oxidation is nearly 38 kJ g^{-1} whereas from carbohydrates and proteins is 17 kJ g^{-1}. Such difference lies in the fact that FAs are much more reduced than carbohydrates or proteins (Berg et al., 2012).

Adipose tissue cells or adipocytes, are specialized cells that act as major site for triacylglycerol storage and synthesis. In mammals, adipose tissue can be found all over the body, particularly under the skin (subcutaneous) and surrounding internal organs (visceral). This tissue works mainly as an energy depot, where droplets of triacylglycerols tend to form large globules that can fill most of cell's volume. The surface of these droplets is covered with perilipins that restrict the access to the lipids (Berg et al., 2012).

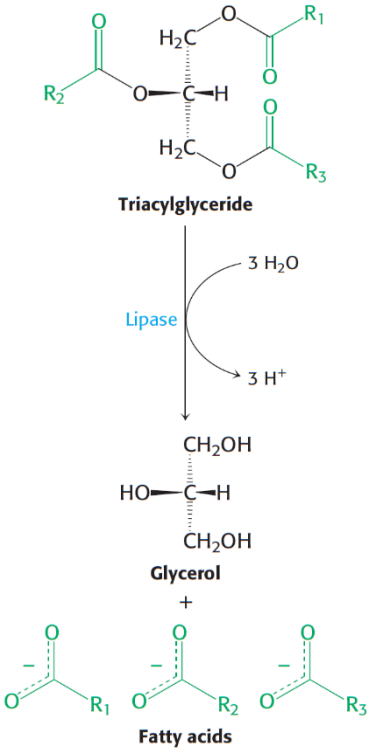
Lipolysis and β-oxidation of free fatty acids (FFAs) is the main energy-yielding pathway for animal tissues. This process occurs in the mitochondria and is characterized by the degradation of FAs to generate acetyl-CoA. Access to the lipid energy reserves stored in adipose tissue involves three main stages: i) mobilization of FFAs; ii) activation and transport to the mitochondria; and iii) degradation into acetyl CoA (Berg et al., 2012; Lehninger et al., 2008).

3.2.1. Mobilization of Free Fatty Acids

The first step consists in isolating the FAs stored as triacylglycerols or mobilizing FFAs. For that, lipases of adipose tissue are activated to hydrolyze triacylglycerols into glycerol and FAs in a process also known as lipolysis (illustration 5).

Adipocyte lipases are activated by hormones such as epinephrine or glucagon. These hormones are responsible for the trigger of seven transmembrane (7TM) receptors that induce adenylate cyclase (EC 4.6.1.1) activity. Increased levels of cyclic adenosine monophosphate (AMP) stimulate protein kinase A (EC 2.7.11.11) activity which, afterwards, activates cytosol hormone-sensitive lipases (EC 3.1.1.79) and perilipins by phosphorylation (Berg et al., 2012; Lehninger et al., 2008).

Activated perilipins are the proteins responsible for: restructuring of the lipid droplet in a way that it's easier for lipases to access triacylglycerols; the release of a coactivator for the adipose triglyceride lipase (ATGL, EC 3.1.1.3). ATGL hydrolyzes a FA forming diacylglycerol in the process, while hormone-sensitive lipase removes a second FA. Monoacylglycerol lipase (EC 3.1.1.23) completes the mobilization producing a last FFA and glycerol (Berg et al., 2012).



Adapted from Berg et al. (2012)

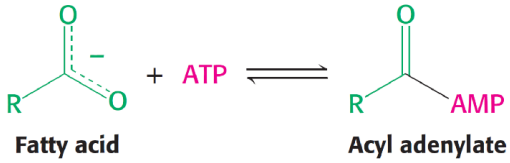
Illustration 5. Hydrolysis of a trigliceride into glycerol and FAs by adipocyte lipases.

Released FFAs are not soluble in blood, so they bind into serum albumin, which will carry them to the energy-requiring tissues. On the other hand, the glycerol is absorbed by the liver where it's transformed into glyceraldehyde 3-phosphate and can participate in either the glycolytic or gluconeogenic pathways (Berg et al., 2012).

3.2.2. Activation and Transport to the Mitochondria

Full animal FA oxidation occurs in the mitochondrial matrix. FA with chain lengths of 12 or fewer carbons do not need membrane transporters to enter in the mitochondria. Longer FFAs need to be activated and then carried by a carnitine based transport mechanism to reach the mitochondrial matrix (Lehninger et al., 2008).

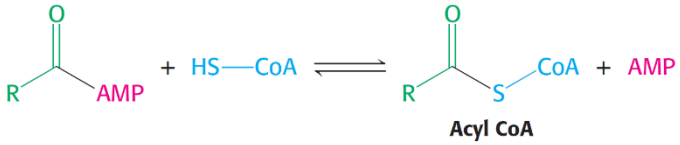
Activation occurs on the outer mitochondrial membrane where long-chain FFAs are linked to Coenzyme A (CoA) by a thioester bond, in a reaction catalyzed by acyl CoA synthetase (EC 6.2.1.3). This process is ATP-dependent and comprehends 2 steps. On the first one, the FA reacts with ATP forming acyl adenylate releasing pyrophosphate (illustration 6) (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Berg et al. (2012)

Illustration 6. Formation of acyl adenylate and pyrophosphate in the first reaction of the FA activation process.

On the second step, the thiol group of CoA displaces AMP, conducing to the formation of the thioester acyl CoA (illustration 7).

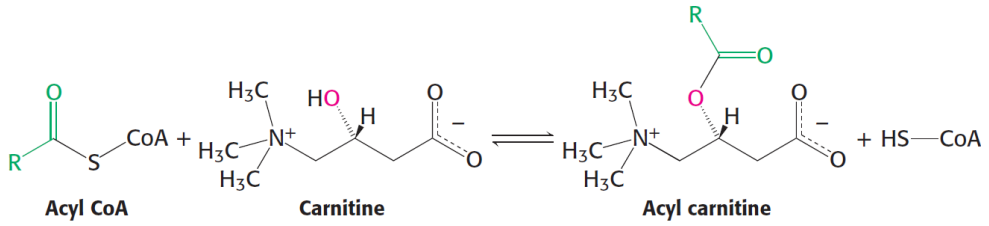


Adapted from Berg et al. (2012)

Illustration 7. The second activation step comprises the formation of the thioester acyl CoA.

Both reactions are reversible and have an equilibrium constant close to 1. In order to pull these reactions in the forward direction, the pyrophosphate formed in the first reaction is rapidly hydrolyzed into two monophosphates by an inorganic pyrophosphatase (EC 3.6.1.1) (Berg et al., 2012; Lehninger et al., 2008).

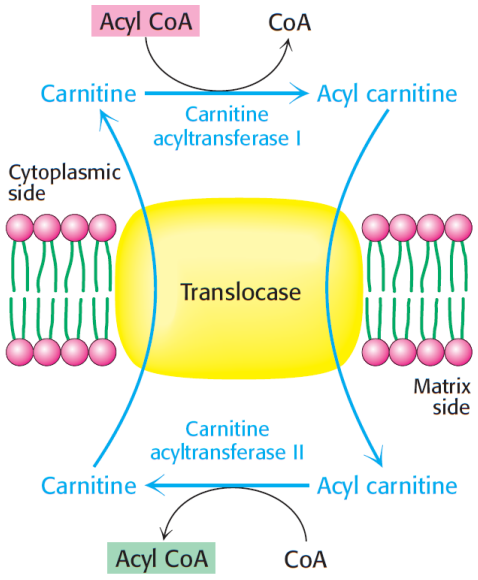
FAs are activated on the outer mitochondrial membrane, yet they are oxidized in the mitochondrial matrix. Their transfer across the mitochondrial membrane involves carnitine. The acyl group present in the activated long-chain FAs binds to the hydroxyl group of carnitine to form acyl carnitine in a reaction catalyzed by carnitine acyltransferase I (EC 2.3.1.21) (Berg et al., 2012; Lehninger et al., 2008) (illustration 8).



Adapted from Berg et al. (2012)

Illustration 8. Transfer of the activated acyl into the mitochondrial matrix involves a previous reaction, on the outer mitochondrial membrane, with carnitine forming acyl carnitine.

Acyl carnitine translocase mediates the entrance of the acyl carnitine ester to the mitochondrial matrix. There, the acyl group is transferred from acyl carnitine to intramitochondrial CoA, in a reaction catalyzed by carnitine acyltransferase II (EC 2.3.1.21), releasing free carnitine. Carnitine can, then, return to the cytosolic side of the mitochondrial membrane through the translocase in exchange for acyl carnitine to balance the process (Berg et al., 2012) (illustration 9).



Adapted from Berg et al. (2012)

Illustration 9. Summary of the acyl group transportation from cytosol to the mitochondrial matrix, a process mediated via acyl carnitine translocase.

3.2.3. Fatty Acid Degradation – β-oxidation

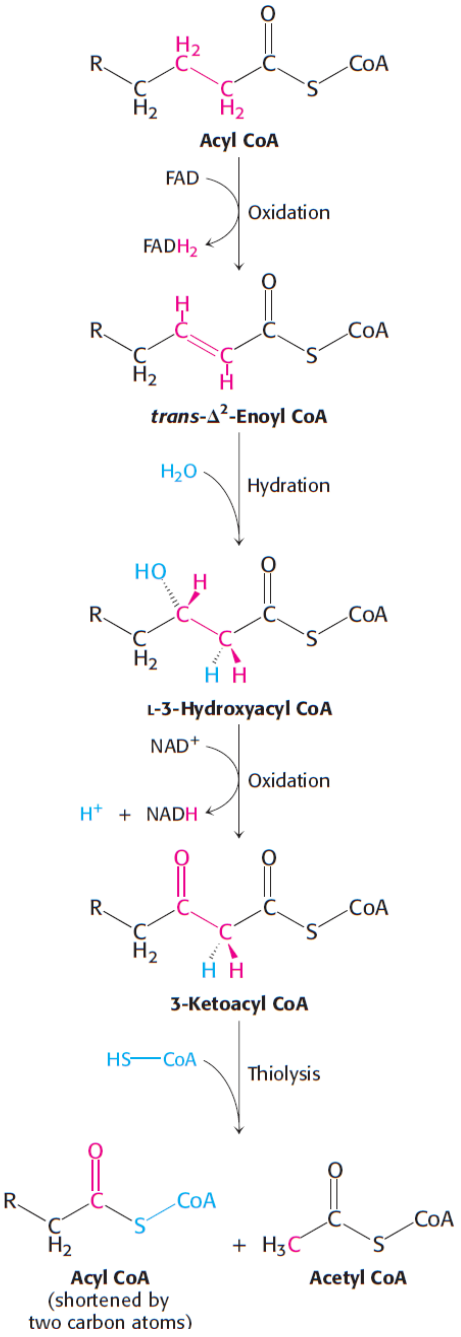
Within the mitochondrial matrix, a four-step operation known as β-oxidation occurs. This cycling oxidation process takes place at the β carbon atom and includes a first oxidation by flavin adenine dinucleotide (FAD), hydration, oxidation by oxidized nicotinamide adenine dinucleotide (NAD^+) and thiolysis by CoA (illustration 10) (Berg et al., 2012).

In the first step, acyl CoA is oxidized to produce a double bond between carbon atoms 2 and 3 in an oxidation process also known as dehydrogenation that is catalyzed by acyl CoA dehydrogenase (EC 1.3.8.7). The lost hydrogen atoms reduce the electron carrier FAD to its reduced form $FADH_2$ (Berg et al., 2012; Lehninger et al., 2008).

The resultant product, trans-Δ^2-enoyl CoA, is then hydrated on the second step conducing to the formation of L-3-hydroxyacyl CoA in a reaction catalyzed by enoyl CoA hydratase (EC 4.2.1.17) (Berg et al., 2012; Lehninger et al., 2008).

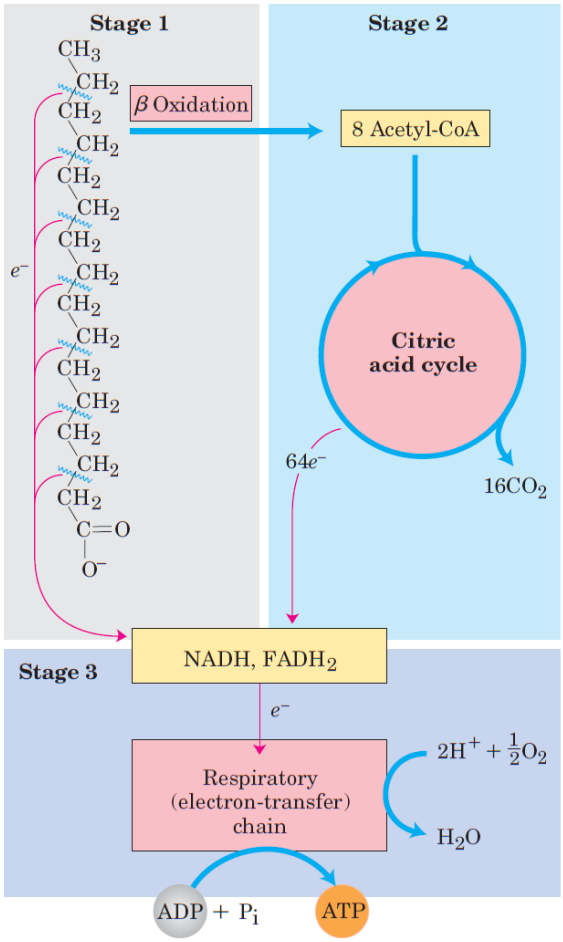
The third step involves another oxidation reaction, which converts the hydroxyl group at C-3 into a keto apart from generating reduced nicotinamide adenine dinucleotide (NADH) from NAD^+. This oxidation is specifically catalyzed by L-3-hydroxyacyl CoA dehydrogenase (EC 1.1.1.35) (Berg et al., 2012; Lehninger et al., 2008).

In the last step (thiolysis), the previously formed 3-ketoacyl CoA is cleaved by the thiol group of a free coenzyme A molecule, yielding acetyl CoA and an acyl CoA molecule shortened by two carbon atoms. This reaction is catalyzed by acyl CoA acetyltransferase (thiolase, EC 2.3.1.16) (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Berg et al. (2012)

Illustration 10. β-oxidation involves 4 main steps: oxidation, hydration, oxidation and thiolysis.



Adapted from Lehninger et al. (2008)

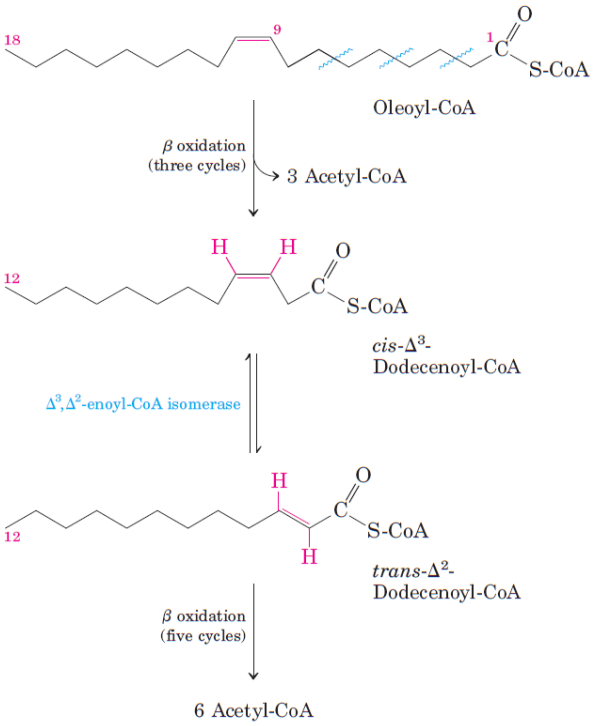
Illustration 11. After β-oxidation, the main resultant product, acetyl CoA, enters the Citric Acid Cycle for e^- production that can be transported via NADH and $FADH_2$ carriers to enter the respiratory chain for ATP synthesis.

Therefore, for each oxidation cycle, a determined acyl CoA is shortened by two carbon atoms and one molecule of $FADH_2$, NADH and acetyl CoA are formed. The last oxidation cycle of an acyl CoA with an even number of carbon atoms ends up producing two copies of acetyl CoA and one molecule of the electron carriers $FADH_2$ and NADH (Lehninger et al., 2008).

The acetyl CoA produced from β-oxidation enters the Citric Acid Cycle (Kreb's Cycle), as well as those derived from glucose via glycolysis, where it's further oxidized to CO_2, while generating more electron carriers such as NADH and $FADH_2$. These, in turn, transfer their energy to the mitochondrial respiratory (electron-transfer) chain, thus inducing the synthesis of more ATP. This can be represented as a three-stage process as shown on illustration 11 (Berg et al., 2012; Lehninger et al., 2008).

3.2.4. Degradation of Unsaturated Fatty Acids

Oxidation of UFAs requires up to two additional reactions compared to the previously described oxidation sequence. The double bonds present in these FAs are frequently in cis configuration, which prevents them from being hydrated on the second degradation reaction. This is because enoyl CoA hydratase, the enzyme responsible for catalyzing the addition of H_20, operates only on trans double bonds. Besides, the presence of a double bond between C-3 and C-4 prevents the formation of another double bond between C-2 and C-3. The solution to this problem comes from cis-Δ^3-enoyl CoA isomerase (EC 5.3.3.8), an enzyme capable of shifting the position and configuration of the cis-Δ^3 double bond to a trans-Δ^2 double bond. The substrate is now similar to those of the SFA oxidation sequence and β-oxidation can proceed (Berg et al., 2012; Lehninger et al., 2008) (illustration 12).

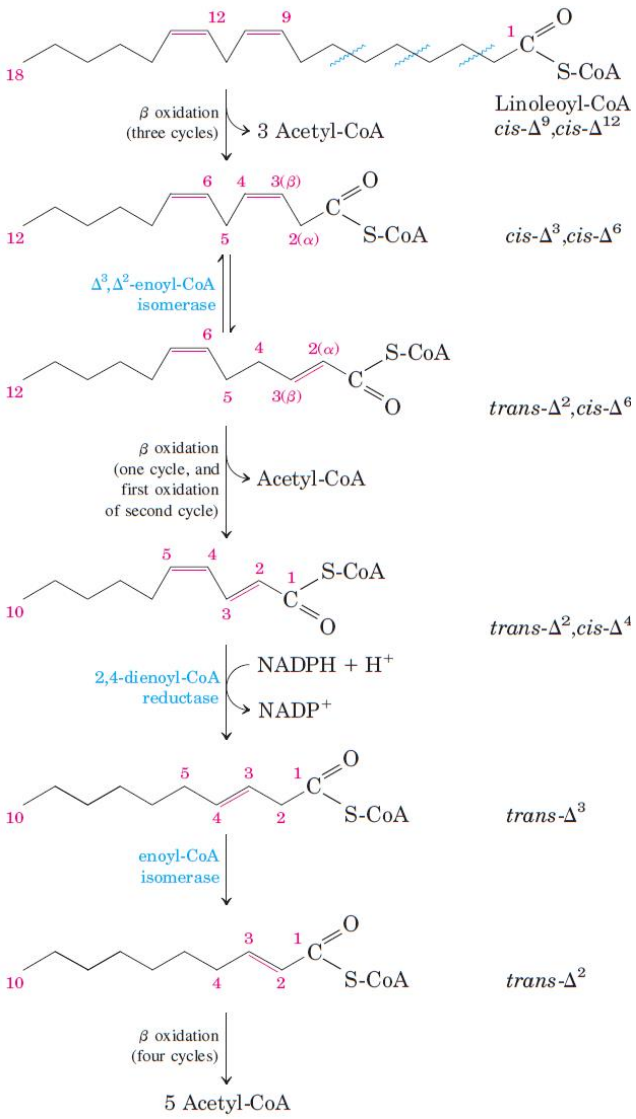


Adapted from Lehninger et al. (2008)

Illustration 12. The full oxidation of a monounsaturated fatty acid (MUFA) involves the action of an isomerase to change the double bond location and configuration.

Polyunsaturated fatty acids (PUFAs), have more than one double bond and require an additional reaction to get fully oxidized.

At some point of the β-oxidation pathway, a stalemate occurs from the formation of a trans-Δ^2, cis-Δ^4-dienoyl CoA intermediate which is not a substrate for trans-enoyl CoA hydratase and therefore, cannot be directly hydrated. Nevertheless, 2,4-dienoyl CoA reductase (EC 1.3.1.34), a NADPH-dependent enzyme can convert this substrate to trans-Δ^3-enoyl CoA. This new substrate can now be converted by cis-Δ^3-enoyl CoA isomerase to trans-Δ^2-enoyl CoA and full oxidation can proceed normally (Berg et al., 2012) (illustration 13).



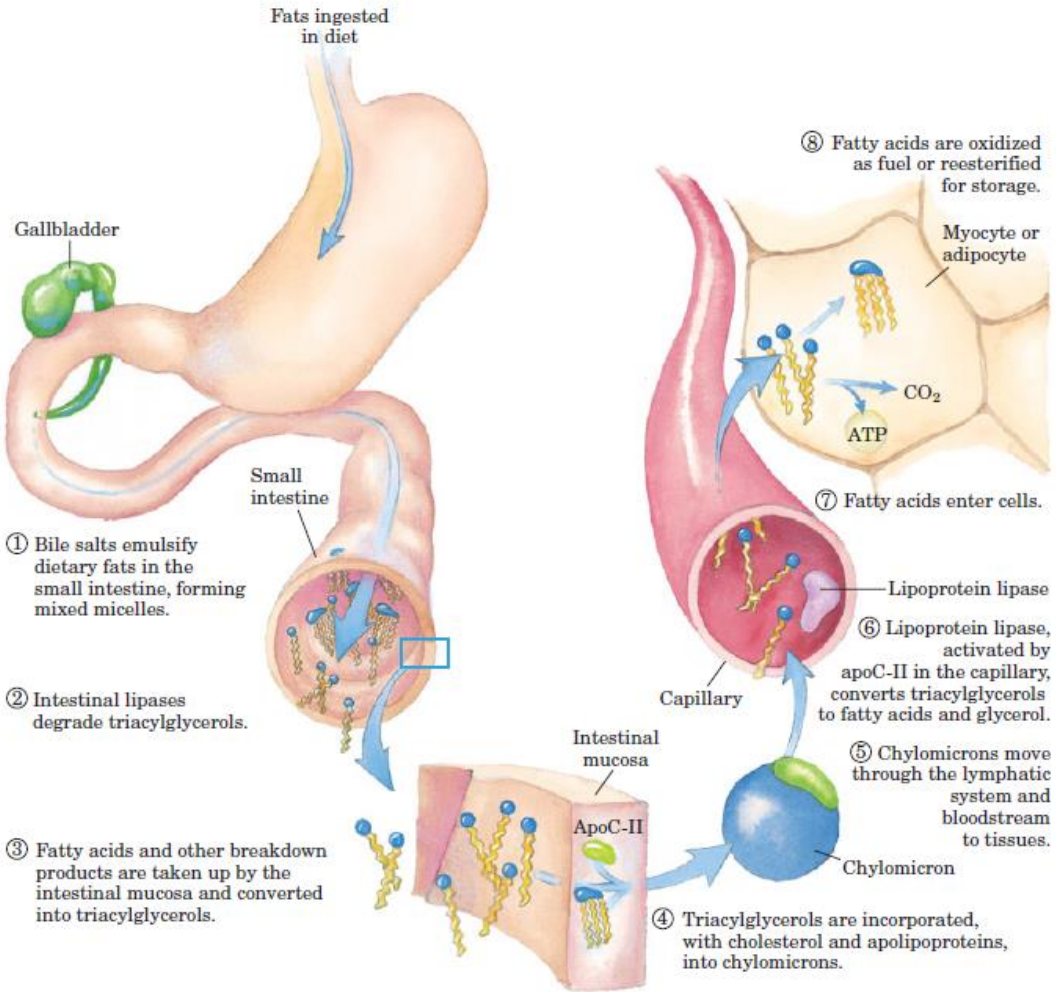
Adapted from Lehninger et al. (2008)

Illustration 13. The full oxidation of a polyunsaturated fatty acid involves the action of an isomerase and a reductase.

3.3. Dietary Lipid Absorption

Most of ingested lipids come in the form of triacylglycerols and must be degraded so they can be absorbed in the intestinal epithelium (illustration 14). Dietary triacylglycerols are emulsified in the small intestine lumen by bile salts, stored in the gallbladder, and hydrolyzed into FFAs and monoacylglycerol by water-soluble intestinal lipases, secreted by the pancreas. These products cross the intestinal mucosa, where they are reconverted to triacylglycerols and are incorporated, along with cholesterol and apolipoproteins, into lipoprotein transport particles known as chylomicrons (Berg et al., 2012; Lehninger et al., 2008).

Chylomicrons are released into the lymphatic system and then into the blood, which transports them to muscle and adipose tissue. On the capillaries, apolipoprotein C-II present in chylomicrons activates lipoprotein lipase, which hydrolyses triacylglycerols to FAs and glycerol. The FAs can then be taken by the target tissue cells, where they can be oxidized for energy (myocyte) or reesterified for storage as triacylglycerols (adipocyte) (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Lehninger et al. (2008)

Illustration 14. Absorption of dietary lipids in vertebrates.

3.4. *De Novo* Synthesis

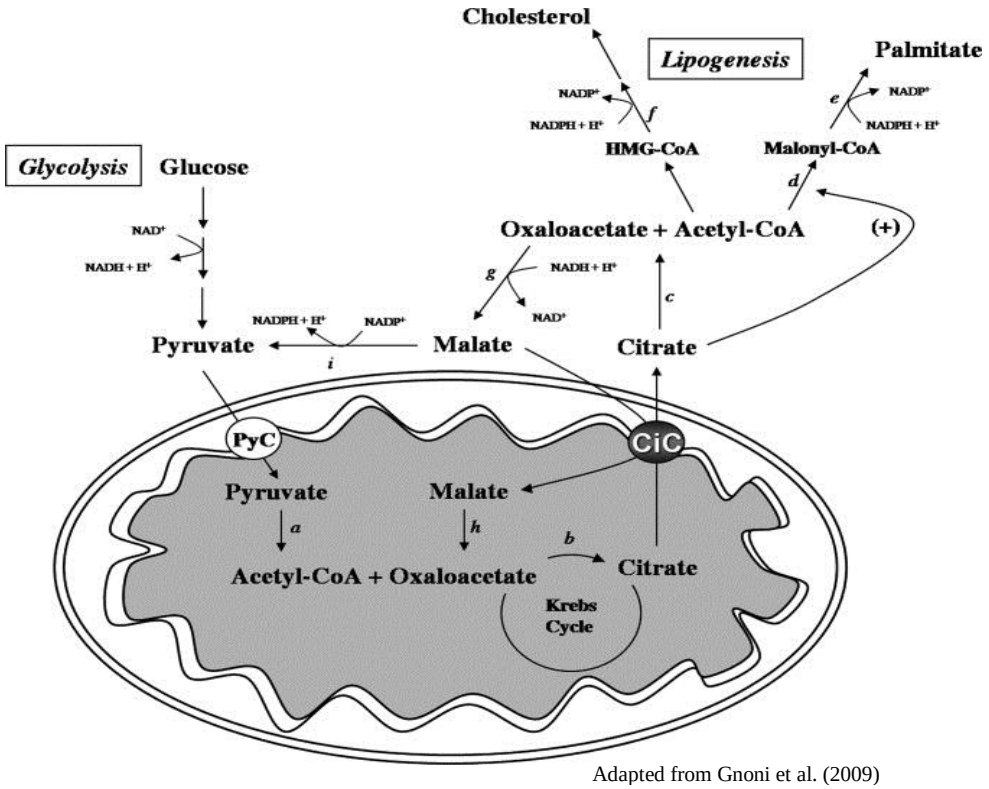
The excess dietary carbohydrates and proteins can be converted/recycled to FAs and then incorporated into triacylglycerols for storage in a process known as *de novo* synthesis. Generally, this endogenous lipogenic pathway for FA biosynthesis becomes unnecessary when the body's lipid requirements are attained in the diet. Nevertheless, this synthesis is particularly important during embryonic development and lactation in mammary glands (Berg et al., 2012; Strable and Ntambi, 2010).

The final product of this synthesis is palmitate, a sixteen carbon SFA that is the precursor for the formation of other FAs in a task involving other enzymatic systems. *De novo* synthesis occurs in the cytoplasm of hepatic, renal and adipose tissue cells besides lactating mammary glands. This process can be divided into two distinct stages: activation of acetyl CoA and the set of reactions of the fatty acid complex (Berg et al., 2012).

3.4.1. Activation – acetyl CoA carboxylation

The end-product of FA degradation, acetyl-CoA, is now the precursor and source of carbons for the synthesis of any FA while the reductant role is played by NADPH molecules. Therefore, the first step of *de novo* synthesis is to obtain acetyl CoA and its further activation (Berg et al., 2012).

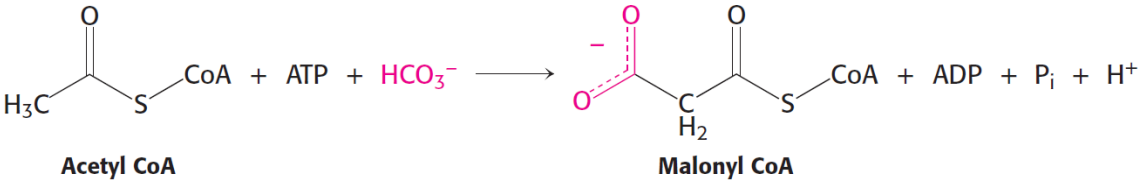
Acetyl CoA is a common organic intermediate product of oxidation of pyruvate, FAs, amino acids and ketone bodies. Nevertheless, the mitochondrial membrane is impermeable to acetyl CoA. Acetyl CoA must be conjugated with oxaloacetate to form citrate, in a reaction catalyzed by citrate synthase (EC 2.3.3.1). Citrate can then be freely transferred to the cytoplasm through the citrate carrier (CiC). These mitochondrial transporters play an important intermediary metabolic role, connecting carbohydrate catabolism and lipogenesis, by facilitating an exchange flow of citrate and malate between the mitochondrial matrix and the cytosol, respectively (illustration 15). In fact, CiC helps citrate get through the permeable inner mitochondrial membrane, which is then transported by passive diffusion from the mitochondrial outer membrane to the cytosol through voltage dependent anion channels. In the cytoplasm, citrate is then cleaved to acetyl CoA and oxaloacetate, in a reaction catalyzed by ATP citrate lyase (EC 2.3.3.8.) In addition, cytosolic citrate can provide, via malic enzyme or NADP malate dehydrogenase (EC 1.1.1.40), reducing equivalents as NADPH molecules that are required in further lipogenesis phases. Nevertheless, most of required NADPH for FA synthesis comes from the pentose phosphate pathway (Gnoni et al., 2009).



Adapted from Gnoni et al. (2009)

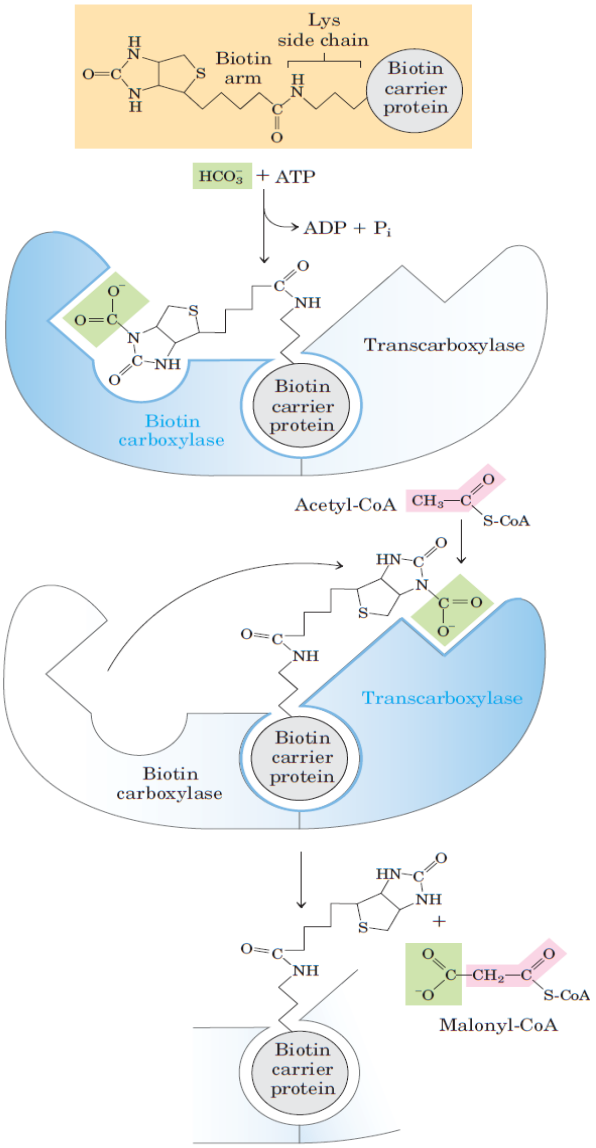
Illustration 15. The metabolic key role played by the CiC providing a linkage between glycolysis and lipogenesis. HMGCoA, 3-hydroxy-3-methylglutaryl-CoA; PyC, pyruvate carrier. (a) Pyruvate dehydrogenase, (b) citrate synthase, (c) ATP-citrate lyase, (d) acetyl-CoA carboxylase, (e) fatty acid synthase, (f) 3-hydroxy-3-methylglutaryl-CoA reductase, (g) cytosolic malate dehydrogenase, (h) mitochondrial malate dehydrogenase, (i) malic enzyme.

Carboxylation of acetyl CoA expresses the beginning of *de novo* synthesis, a reaction catalyzed by acetyl CoA carboxylase (ACC). This process is irreversible and results in the formation of the substrate malonyl CoA (illustration 16). ACC in animals is a single multifunctional polypeptide that has three functional domains (see illustration 17): the biotin carboxylase, which activates a carboxyl group derived from hydrogencarbonate in an ATP-dependent reaction; the biotin carrier protein, which accepts the activated carboxyl group binding it to a nitrogen in the biotin ring; and the transcarboxylase, which is responsible for the transfer of the activated carboxyl group from the carboxybiotin intermediate to acetyl CoA yielding malonyl CoA (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Berg et al. (2012)

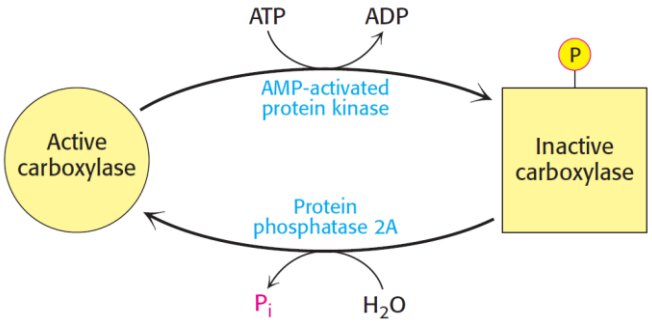
Illustration 16. The first step of *de novo* synthesis comprises the carboxylation of acetyl CoA into malonyl CoA.



Adapted from Lehninger et al. (2008)

Illustration 17. The acetyl CoA carboxylase is a three subunit polypeptide that mediates the carboxylation of acetyl CoA to malonyl CoA.

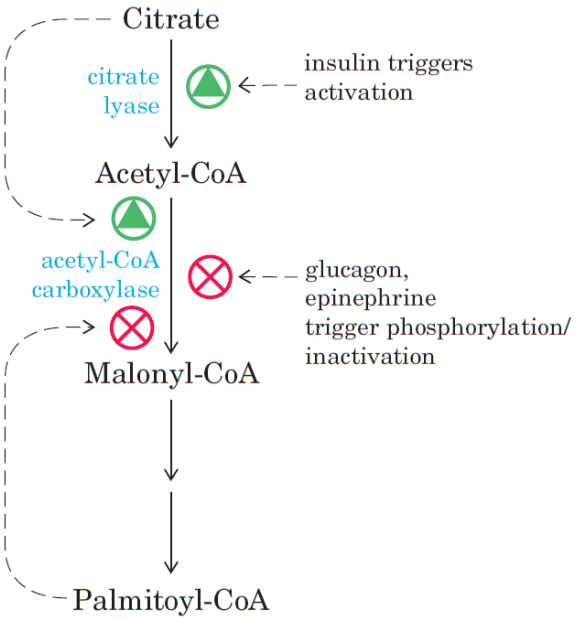
This reaction is the rate-limiting step in FA synthesis, with ACC playing a key role in controlling the balance between oxidation and synthesis in the metabolism of FAs. Inactivation of ACC occurs by phosphorylation in an ATP-dependent reaction catalyzed by AMPK that dissociates the polypeptide into monomeric subunits which causes loss of activity (see illustration 18). AMPK works has a fuel gauge since it's activated by AMP and inhibited by ATP. Therefore, in circumstances of low cellular energy levels, cells tend to stop synthesizing new lipids. On the other hand, protein phosphatase 2A (EC 3.1.3.16) catalyzes the inverse reaction, the activation of ACC by dephosphorylation (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Berg et al. (2012)

Illustration 18. Regulation of acetyl CoA carboxylase by phosphorylation/dephosphorilation.

The flow of precursors into malonyl CoA can also be regulated by hormones and substrates (illustration 19). Citrate stimulates allosterically ACC activity. High levels of citrate induce polymerization of inactive subunits that can partly reverse the inhibition produced by phosphorylation. On the other hand palmitoyl CoA, the main product of FA synthesis, inhibit ACC activity by causing the same effects as phosphorylation, dissociating the ACC polypeptide into inactive subunits. Palmitoyl CoA also limits FA synthesis by inhibiting CiC, preventing the entry of citrate from the mitochondria into the cytosol. Glucagon and epinephrine, under conditions of fasting and exercise, stimulate the mobilization of FAs from triacylglycerols in cell depots which will be used as urgent energy source. These hormones also inhibit FA synthesis by inhibiting acetyl CoA carboxylase and though the exact mechanisms are not yet entirely understood, it is recognized that they enhance the inhibition provoked by the activity of AMPK by phosphorylating ACC (Berg et al., 2012; Lehninger et al., 2008).



Adapted from Lehninger et al. (2008)

Illustration 19. Control on the FA biosynthesis by hormonal triggers and other substrate factors.

FA synthesis and oxidation are opposite operations that, if performed simultaneously, would result in a nonsense wasting energy strategy. Therefore, these two major lipid metabolic processes have to be permanently and tightly regulated. For example, the first intermediate product of the activation of acetyl CoA in *de novo* synthesis, malonyl CoA, is responsible for inhibiting the key transporter in β-oxidation, carnitine acyltransferase I, preventing the formation of the intermediate acyl carnitine (Berg et al., 2012; Lehninger et al., 2008).

3.4.2. Reactions of the Multi Enzymatic Fatty Acid Synthase (FAS) Complex

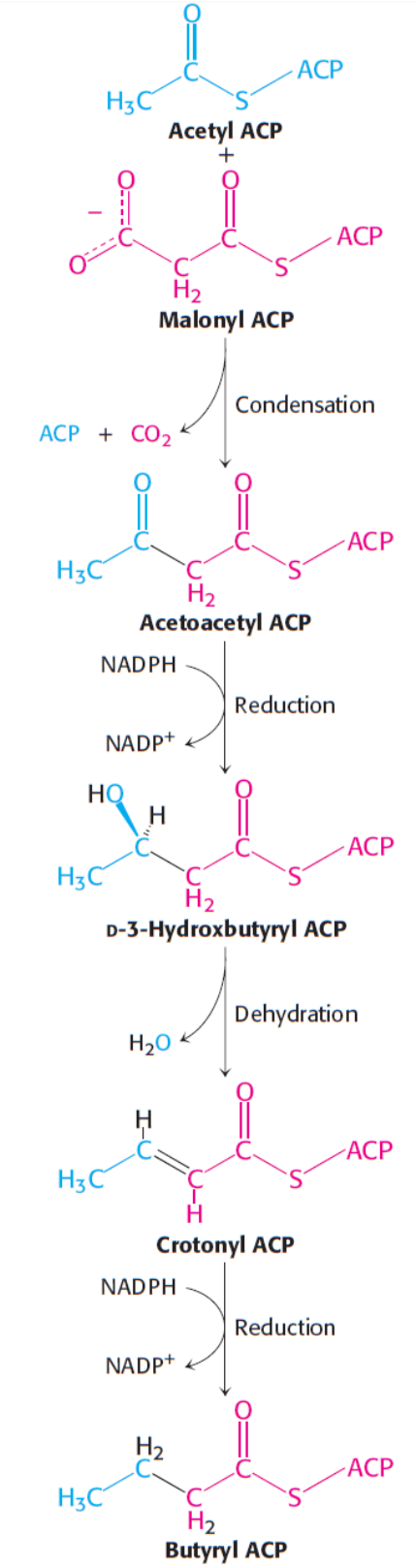
Long carbon chain FAs are assembled in multiple repeated four-step cycles comprehending: condensation, reduction, dehydration, and reduction (see illustration 20). Each cycle yields two carbon atoms to the acyl chain, which is the substrate for the next cycle with an activated malonyl CoA group. For the complete synthesis of palmitate (C16:0) the cycle must be repeated seven consecutive times (Berg et al., 2012; Lehninger et al., 2008).

These reactions are operated by a multifunctional enzyme complex, fatty acid synthase (FAS, EC 2.3.1.85). This enzyme is a dimer of identical 270 kDa subunits. Each subunit presents a set of seven successive enzymes and a protein, acyl carrier protein (ACP), that together make up a fully functional enzymatic system capable of synthesizing a 16 carbon SFA (Berg et al., 2012; Lehninger et al., 2008).

ACP is responsible for coordinating and carrying the reaction intermediates throughout the several enzymes active sites in each cycle. The bond of ACP to the intermediates is similar to the one that occurs in FA degradation between CoA and the intermediates. On both cases, the intermediates are linked to the sulfhydryl terminus of a phosphopantetheine group. In ACP this group is attached to a serine residue (Berg et al., 2012; Lehninger et al., 2008).

Elongation stage starts with the linkage of acetyl CoA and malonyl CoA to ACP. This reaction, catalyzed respectively by acetyl transacylase and malonyl transacylase (or malonyl-acetyl transferase), results in the formation of acetyl ACP and malonyl ACP. In both reactions coenzyme A is released (Berg et al., 2012; Lehninger et al., 2008), as we can see in the following reactions:

$$\text{Acetyl CoA} + \text{ACP} \rightleftharpoons \text{acetyl ACP} + \text{CoA}$$
$$\text{Malonyl CoA} + \text{ACP} \rightleftharpoons \text{malonyl ACP} + \text{CoA}$$



Adapted from Berg et al. (2012)

Illustration 20. The basic steps of one cycle in FA synthesis.

The condensation reaction is catalyzed by β-ketoacyl synthase and comprises the formation of acetoacetyl ACP from the reaction of acetyl ACP with malonyl ACP where CO_2 is also released (Berg et al., 2012; Lehninger et al., 2008):

$$\text{Acetyl ACP} + \text{malonyl ACP} \longrightarrow \text{acetoacetyl ACP} + \text{ACP}$$

This reaction is known as condensation reaction because it results in the formation of a four carbon compound from a two and three carbon units, acetyl ACP and malonyl ACP, respectively (Berg et al., 2012).

In the next step, acetoacetyl ACP undergoes reduction of the carbonyl group at C-3 to form D-3-hydroxybutyryl ACP, a reaction catalyzed by β-ketoacyl reductase where NADPH acts as an electron donor. This reaction opposes the one occurring in FA degradation and exemplifies the principle that NADPH is consumed in biosynthetic reactions and NADH is generated in energy-yielding reactions (Berg et al., 2012).

The third step requires the deletion of water elements present in C-2 and C-3 of D-3-hydroxybutyryl ACP to yield a double bond, resulting in the formation of trans-Δ^2-enoyl ACP or crotonyl ACP. This dehydration reaction is catalyzed by 3-hydroxyacyl-ACP dehydratase (Berg et al., 2012; Lehninger et al., 2008).

The completion of one cycle is concluded with a second reduction reaction in the double bond location of crotonyl ACP, which is reduced to butyryl ACP. This reaction is catalyzed by enoyl ACP reductase and again uses NADPH as reductant (Berg et al., 2012; Lehninger et al., 2008).

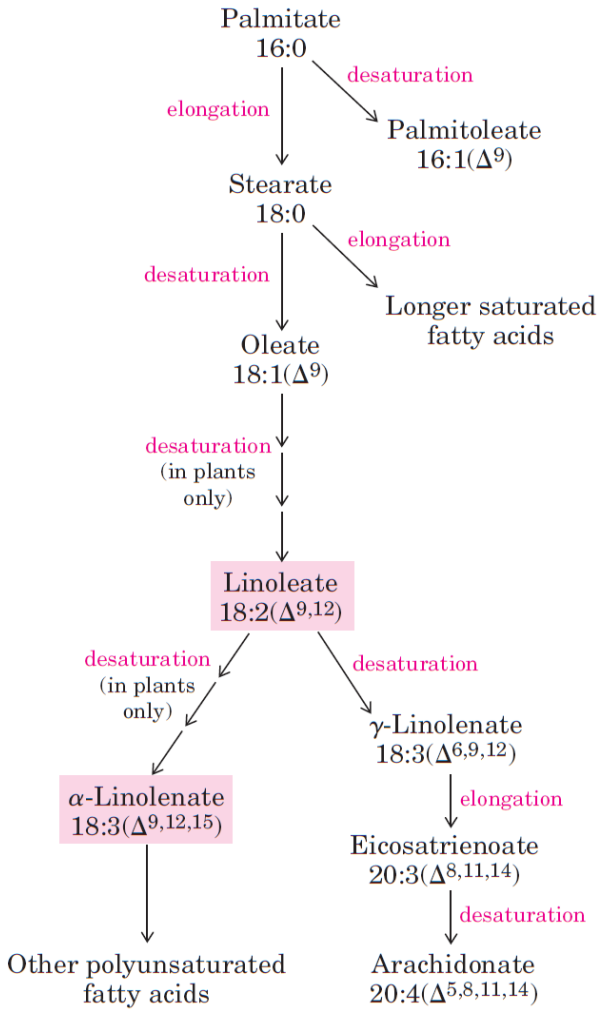
The resultant four carbon compound of the first round of FA synthesis, butyryl ACP, is the main substrate for the second cycle that starts by condensing it with malonyl ACP to form C_6-β-ketoacyl ACP, a reaction much similar to the one in the first cycle. The elongation cycles continue until C_{16} acyl ACP is produced and, at that moment, a thioesterase hydrolyzes this intermediate producing palmitate and ACP. One molecule of H_2O is consumed to break this bond (Berg et al., 2012; Lehninger et al., 2008).

The overall reaction for the synthesis of palmitate, from acetyl CoA already present in the cytosol, can be expressed by the following equation (Berg et al., 2012):

$$8\ \text{acetyl CoA} + 7\ \text{ATP} + 14\ \text{NADPH} + 14\ \text{H}^+ \longrightarrow \text{Palmitate} + 8\ \text{CoA} + 7\ \text{ADP} + 7\ \text{P}_i + 14\ \text{NADP}^+ + 6\ \text{H}_2\text{O}$$

3.5. Biosynthesis of Long-Chain SFAs, MUFAs and PUFAs

Further elongation and unsaturation of FAs are achieved by accessory enzymatic systems on the precursor palmitate (illustration 21). Longer FAs such as stearate (C18:0) and others are produced in reactions catalyzed by enzymes located in the cytoplasmic face of the endoplasmic reticulum membrane. These enzymes, known as elongases, are responsible for adding two-carbon units to the carboxyl ends of the acyl substrates. In these reactions, CoA rather than ACP is the acyl carrier, but the sequence of events is similar to that in palmitate synthesis. Condensation occurs again by decarboxylation of malonyl CoA that remains the two-carbon donor which precedes reduction, dehydration and reduction (Berg et al., 2012; Hussain et al., 2013; Lehninger et al., 2008).



Adapted from Lehninger et al. (2008)

Illustration 21. Biosynthesis of MUFAs and PUFAs derive from palmitate synthesis with accessory enzyme systems. FAs shaded pink cannot be produced by mammals and have to be ingested in the diet.

Working cooperatively with elongases in the production of various MUFAs and PUFAs are another group of enzymes known as desaturases (EC 1.14.19). These enzymes catalyze the introduction of a C-C double bond in different locations of the aliphatic chain of an acyl CoA substrate (Berg et al., 2012; Hussain et al., 2013; Lehninger et al., 2008).

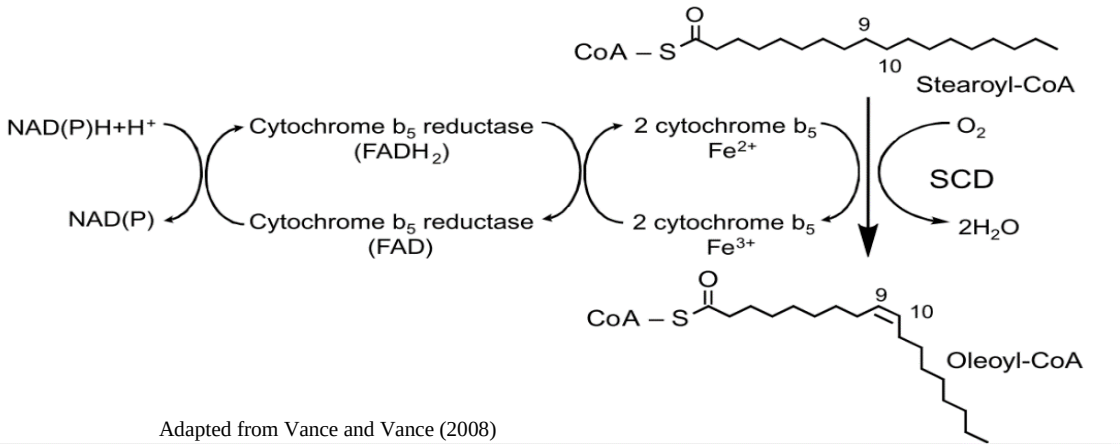
In humans, for example, four families of desaturases are fully documented: Δ^9 desaturase (EC 1.14.19.1), mainly responsible for the production of two of the most common MUFAs, palmitoleic and oleate, establishing a double bond between C-9 and C-10; and Δ^4, Δ^5, Δ^6 desaturases which are responsible for introducing double bonds in the carbon positions 4, 5 and 6, respectively (Hussain et al., 2013).

MUFAs are formed in mammalian systems by direct oxidative desaturation in which the removal of two hydrogen results in the formation of a double bond (Berg et al., 2012; Lehninger et al., 2008; Vance and Vance, 2008).

Conversion of stearoyl CoA into oleoyl CoA, for example, is a complex process mediated by three proteins located in the endoplasmic reticulum membrane (NADH-cytochrome b_5 reductase, cytochrome b_5 and a desaturase) that requires an electron donor such as NADH or NADPH and molecular oxygen (Berg et al., 2012; Lehninger et al., 2008; Vance and Vance, 2008):

$$\text{stearoyl CoA} + \text{NADH} + H^+ + O_2 \longrightarrow \text{oleoyl CoA} + NAD^+ + 2\ H_2O$$

The reaction involves an electron transport system that starts by the transfer of electrons from NADPH to the FAD present on the enzyme NADH-cytochrome b_5 reductase, reducing it to $FADH_2$ (illustration 22). The heme iron (III) atom of cytochrome b_5 can then be reduced to iron (II) which provokes a conversion of the nonheme iron (III) atom of the desaturase, in this case Δ^9 desaturase or stearoyl CoA desaturase (SCD), into iron (II). SCD can now react with O_2 and the saturated acyl CoA substrate that in this case is stearoyl CoA. Besides the formation of a double bond in C-9 present in oleoyl CoA, this reaction also releases two H_2O molecules. In this desaturation process, the rate-limiting factor is SCD (Berg et al., 2012; Lehninger et al., 2008; Ren et al., 2004; Vance and Vance, 2008).



Adapted from Vance and Vance (2008)

Illustration 22. The desaturation of a FA involves an electron-transport chain.

MUFAs synthesized by SCD, namely palmitoleate (16:1, Δ^9) and oleate (18:1, Δ^9), are then used as substrates for the synthesis of various lipid classes including phospholipids, triacylglycerols and cholesteryl esters (Vance and Vance, 2008).

Mammalian hepatocytes cannot establish double bonds beyond the C-9 position of fatty acids. Therefore mammals cannot convert linoleate (18:2, $\Delta^{9, 12}$), or α-linolenate (18:3, $\Delta^{9, 12, 15}$) which are required in the diet as essential FAs because they are necessary precursors for the synthesis of other products (Hussain et al., 2013; Lehninger et al., 2008).

4. Fatty Acid Synthase

4.1. Structure and Activity of the Mammalian FAS Complex

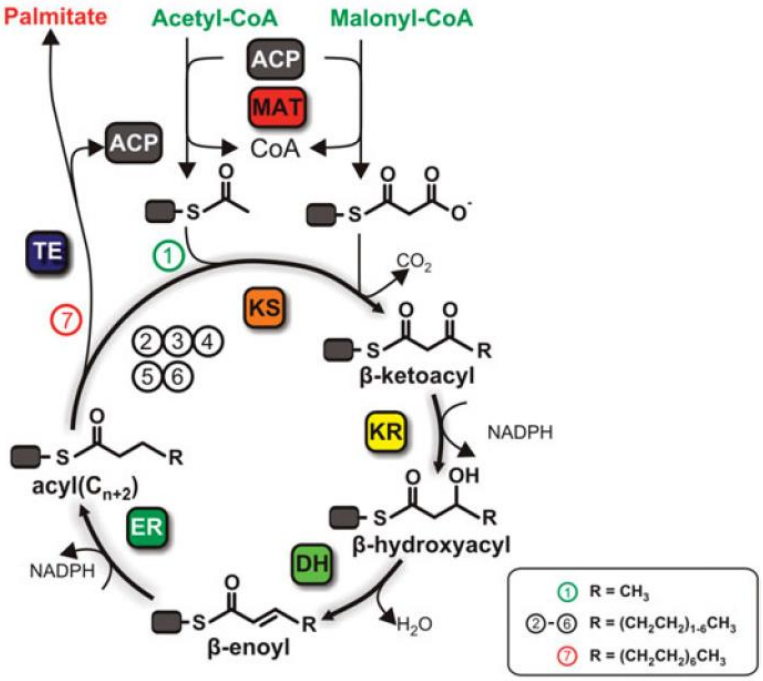
Fatty acid synthase multienzyme complex is a dimer composed of identical 270-kD multifunctional polypeptides that occurs in living organisms in two distinct types, FAS I and FAS II. The first can be found in vertebrates and fungi while the latter is present on plants and bacteria (Lehninger et al., 2008).

While in type II systems every catalytic function is provided by individual enzymes, in type I all necessary catalytic centers are aggregated in a single polypeptide chain. In the mammalian FAS I, for example, seven active sites for different reactions lie in separate domains, with each catalytic site participating in the last step of *de novo* synthesis (illustration 23). Therefore, within each monomeric subunit, seven active domains can be found: malonyl-acetyl transferase (MAT); ketoacyl synthase (KS); ketoacyl reductase (KR); dehydratase (DH); enoyl reductase (ER) and thioesterase (TE), besides ACP for transport of acyl conjugates throughout each catalytic center (Berg et al., 2012; Maier et al., 2008) (illustration 24).

The overall swine FAS structure described by Maier et al. (2008) identifies two additional nonenzymatic domains: a pseudo-ketoreductase and a peripheral pseudo-methyltransferase. These residual structures are thought to be of an ancestral methyltransferase domain that can still be found in polyketide synthases, a class of multienzymatic complexes that produce secondary metabolites known as polyketides such as erythromycin. Nevertheless, the ACP and TE domains are still undetermined in pigs.

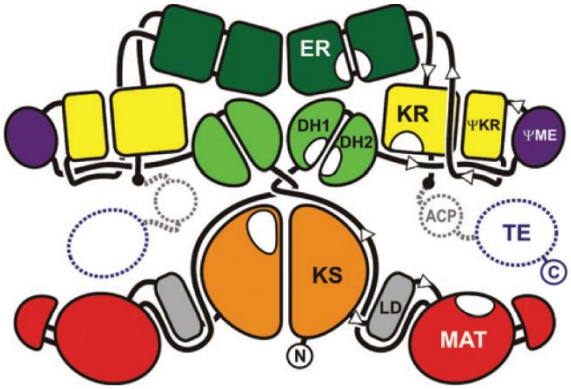
Each monomer adopts a coiled conformation allowing multiple intra- and inter-monomer functional domain interactions with the KS domains located in the central core of the structure. In mammals, MAT is a bifunctional protein domain that catalyzes the

transfer of malonyl and acetyl groups from malonyl CoA and acetyl CoA to ACP, respectively (Maier et al., 2008).



Adapted from Maier et al. (2006)

Illustration 23. Final cyclic reaction in palmitate synthesis mediated by FAS complex.



Adapted from Maier et al. (2008)

Illustration 24. Front view and linear organization schemes of the mammalian FAS with respective catalytic sites colored. Linker domains (LD) are highlighted in gray. DH1 and DH2 represent two dehydratase modules, however, only one is active.

As shown in chapter 3, FAS plays a central role in energy homeostasis by converting excess dietary consumption into storage lipids that can later provide energy via β-oxidation.

FAS activity can be found almost in every tissue, though higher enzymatic expression levels can only be found within lipogenic tissues, namely in adipocytes, hepatocytes and lactating mammary glands (Jayakumar et al., 1995).

In humans, its expression is upregulated by diet carbohydrates, particularly by simple sugars. This occurrence is particularly noticeable after a fasting period. This regulation occurs mostly at a pretranslational phase by controlling mRNA levels. This stimulation is highly associated with insulin since FASN mRNA levels do not increase in diabetic rats and, on the contrary, an increase on FASN expression can be found in hyper-insulinemian rat livers. On the other hand, glucagon suppresses FASN transcription through cAMP-dependent mechanisms (Hopperton, 2012; Semenkovich, 1997).

Fatty acid synthesis in the liver and adipose tissues is strongly decreased by small amounts of exogenously supplied fatty acids. Furthermore, dietary/exogenous PUFAs decrease FAS expression, particularly alpha-linolenic (C18:3, $\Delta^{9, 12, 15}$), docosahexaenoic (C22:6, $\Delta^{4, 7, 10, 13, 16, 19}$), eicosapentaenoic (C20:5, $\Delta^{5, 8, 11, 14, 17}$) and arachidonic (C20:4, $\Delta^{5, 8, 11, 14}$), which have been shown to suppress FAS expression in the liver and adipose tissue (Hopperton, 2012; Semenkovich, 1997).

In non-lipogenic tissues, FASN expression is modulated through hormonal signaling. Besides insulin and glucagon, thyroid hormone and glucocorticoids increase FASN expression. Furthermore, estrogen and progesterone are also associated with elevated FASN expression. Transcription factors such as sterol regulatory element binding protein 1-c (SREBP1-c) mediate the regulation of FAS transcription by hormonal signals (Hopperton, 2012; Semenkovich, 1997).

Various compounds have been discovered and designed to inhibit FASN expression with the particular interest as anti-obesity drugs and for antitumoral treatments. Cerulenin and its derivatives, particularly C75 and C93, are small designed molecules that have showed inhibitory effects on FASN, by targeting the ketoacyl synthase domain, besides significant antitumoral effects. Orlistat is an FDA approved anti-obesity drug that inhibits FASN expression, by binding and inhibiting the thiosterase domain activity. Other inhibitors of FASN activity include epigallocatechine-3-gallate (EGCG) and other natural flavonoids (Hopperton, 2012; Liu, 2008; Loftus et al., 2000).

Clop et al. (2003) proposed FASN as candidate gene for FA composition. Ever since, more and more studies have pointed out the potential influence of this gene SNPs on porcine FA composition and meat quality (Corominas et al., 2013; Kim et al., 2011; Maharani et al., 2012; Munoz et al., 2007; Munoz et al., 2003).

Other authors as Berndt et al. (2007) propose FASN to be as well a candidate gene for the study of metabolic disorders as obesity and type 2 diabetes because of its central role in FA lipogenesis. They have shown that increased FASN expression in adipose tissue is associated to excess energy intake and accumulation of body fat. Furthermore, they state that adipose FASN mRNA expression might significantly mislead insulin sensitivity and circulating adipokine patterns.

4.2. FAS gene sequence and polymorphisms

Eukaryotic genes are organized in exons and introns. Exons are the sequences that encode for the protein, while introns are non-expressed sequences that get removed during maturation of pre-mRNA.

Genetic polymorphisms on coding sequences (exons) are occurrences that induce genetic variation, adaptation and biodiversity. SNPs are the most common genetic variations in which a single nucleotide is substituted, deleted or added in the transcribed sequence. These polymorphisms may affect the overall expression of the triplet (non-synonymous SNPs) or not (synonymous SNPs) (Kimball, 1994).

FASN is a highly conserved gene among mammals. Swine (*Sus scrofa*) FASN (accession number NM_001099930.1) shares 85% of identity with that of the cow (*Bos taurus*) (accession number: NM_001012669.1) and of the goat (*Capra hircus*) (accession number DQ223929.1), 83% with humans (*Homo sapiens*) (accession number NM_004104.4) and 79% with mouse (*Mus musculus*) (accession number AF127033.1) with 93% sequence coverage.

The main objective of this work was to obtain the coding FASN sequence in AL pigs in subcutaneous adipose tissue and to compare it with that of the domestic pig (*Sus scrofa*). To our knowledge, there is no information regarding the genomic organization of genes of lipogenic enzymes in the obese AL pig, including fatty acid synthase. Bibliographic references are also scarce.

III. MATERIAL AND METHODS

1. Slaughter conditions and tissue collection

The purebred Alentejano pig from where biological samples were collected was fed a commercial diet (Proibérico 2, Provimi, Alverca, Portugal). The specific composition of this diet is proprietary information. The commercial diet provided 150 g/kg crude protein, 30 g/kg crude fat (with ~60% of oleic and linoleic acids), and 13.0 MJ digestible energy.

Samples were collected from one purebred AL pig (P) in an industrial slaughterhouse. This animal was killed at 100 kg BW by electronarcosis and bleeding. Tissue samples were obtained from subcutaneous adipose tissue, snap frozen in liquid nitrogen and stored at -80 ºC until analysis.

2. RNA extraction

Total RNA extraction from the collected subcutaneous adipose tissue (SUB) was performed using the commercial GeneJET™ RNA Purification Kit (Thermo Scientific). Up to 100 mg of tissue were disrupted using a conventional rotor-stator homogenizer, Precellys® Minilys (Bertin Technologies), in a 10 s at 5000 rpm two cycle program with a 10 s rest time between cycles, following the manufacturer's instructions. Assessment of total RNA integrity was performed using a NanoDrop® 2000 Uv-Vis Spectrophotometer (Thermo Scientific) at 260 nm and purified RNA was stored at -80 ºC until use.

3. Complementary DNA synthesis

The first strand cDNA synthesis reaction, namely Reverse Transcription (RT) reaction, was performed according to Maxima® First Strand cDNA Synthesis Kit for RT-qPCR reactions (Thermo Scientific). This commercial kit produces cDNA with both oligo $(dT)_{18}$ and random hexamer primers. The resulting cDNA product was stored at -20 ºC until usage.

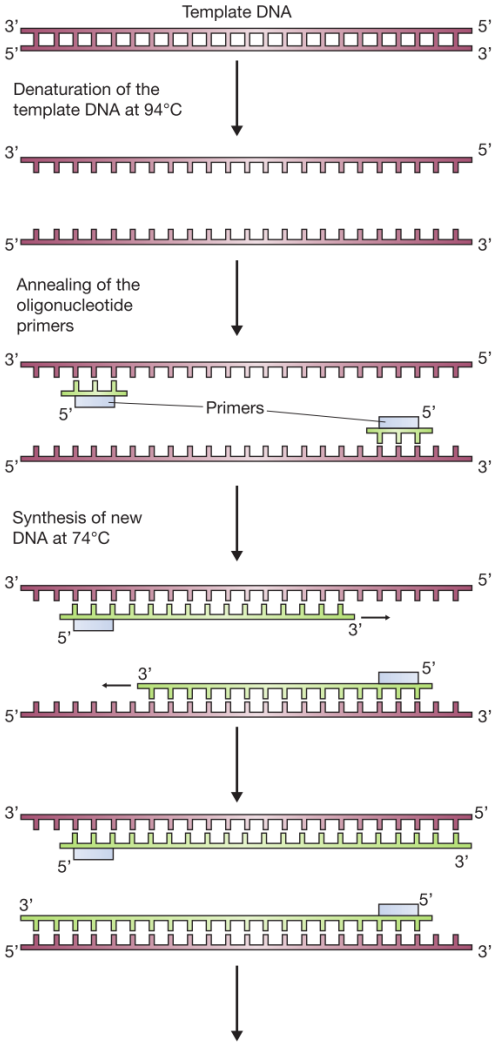
4. cDNA Amplification by PCR

Amplification by polymerase chain reaction (PCR) enables a fast and convenient acquirement of multiple specific DNA pieces, set by a pair of oligonucleotide primers that flank the target sequence region.

Briefly, PCR reactions stand for multiple repeated cycles of heating, cooling and heating, allowing denature of double stranded DNA, binding of primers and synthesis of complementary DNA by a *Taq* polymerase, respectively, that generate countless double stranded target DNA sequence molecules (illustration 25).

In RT-PCR in particular, the first PCR cycle starts with denaturation of the molecule produced in reverse transcription, a double stranded molecule of RNA and complementary DNA, therefore, only one DNA template is yielded.

All PCR reaction were programmed and performed in a MyCycler™ Thermal Cycler (Bio-Rad).



Adapted from Brown (2010)

Illustration 25. The basic steps in polymerase chain reaction; DNA denaturation, primer annealing and extension.

4.1. Sequencing Strategy

The *Sus scrofa* FASN complete coding sequence (cds) was retrieved from NCBI database, with the reference number NM_001099930.1 as guidance for the AL pig sequencing strategy. Then, specific target regions were conceived for consecutive partial sequencing of the gene based on sets of primers available on literature and newly designed ones (illustration 26). For amplification and cloning of the 5' and 3' ends, rapid amplification of cDNA end's (RACE) technique was performed.

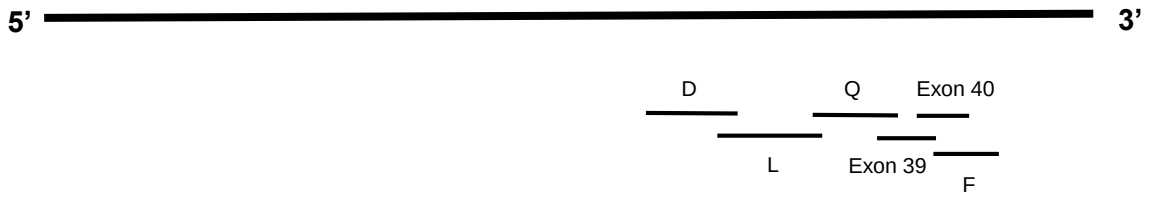


Illustration 26. Partial sequencing strategy for the fatty acid synthase gene.

4.2. Primer Design

Little was found in current literature about *Sus scrofa* FASN sequencing strategies and specific primer sequences, nevertheless, five pairs of previously described primer sequences were fully tested, namely FAS S/AS (Huang et al., 2007), FASN1-FW/FASN1-RV and FASN2-FW/FASN2-RV (Munoz et al., 2003), FASN39-FW/FASN39-RV and FASN40-FW/FASN40-RV (Kim et al., 2011). Still, only the latter two pairs gave products successfully amplified and sequenced.

Since there weren't much swine FASN primer sequences available in current literature, several sets of primer pairs were designed using specific Biosoftware tools such as Primer3Web version 4.0.0, NetPrimer (PREMIER Biosoft) and BioEdit Sequence Alignment Editor Version 7.1.7. All primers that successfully amplified FASN gene sequences are listed and described on table 2. For a complete list of tested primers check appendix I (Hall, 1999; Untergasser et al., 2012).

Table 2. Complete list of primers used in FASN sequencing along with respective sequence, gene location regarding to swine FASN record NM_001099930.1 on GenBank, annealing temperature and amplified product size.

Primer Designation	Gene Location (bp)	5'→ 3' Sequence	Annealing Temperature (ºC)	Amplified Product size (bp)
FAS D FW	4881-4902	CCACCTCTGTTCTGCTGCTTCA	65	685
FAS D RV	5546-5565	CAGGGCAAGCACATAGGCAA		
FAS L FW	5393-5415	ATCCTGCTGGACTCGCTCTTTGA	65	783
FAS L RV	6158-6175	CGGCACGACGGGCTCCCA		
FAS Q FW	6108-6130	CCAACTACGGCTTCGCCAACTCT	65	660
FAS Q RV	6746-6767	CGATGGGGTGGACCAGGAACAG		
FASN exon39-FW	6615-6631	CGGCGACTCCCACATCC	60	431
FASN exon39-RV	7025-7045	GAAGGTGTGTGAGCCGTCGAA		
FASN exon40-FW	6908-6928	CCCGAGGGGCCTTACCGCATC	60	378
FASN exon40-RV	7268-7285	GAAGGAGCGAGCGGCGAA		
FAS F FW	7015-7035	CCTCTTCCTGTTCGACGGCTC	58	497
FAS F RV	7490-7511	TGTGGATGATGCTGAGGATGGA		

4.3. PCR Protocol Optimization

PCR reactions were performed with multiple conditions and reagents in PCR specific tubes.

DNA fragment "D" amplification was assembled with: 0.3 mM of a deoxynucleotide triphosphates (dNTPs) mixture, 0.2 µM of each primer, 50 ng of DNA template, 1x DreamTaq buffer (containing optimized concentrations of KCl and $(NH_4)_2SO_4$ and 2 mM $MgCl_2$) and 2.5 U of DreamTaq DNA Polymerase (Thermo Scientific) in a total reaction volume of 50 µL. The amplification program executed was: 94 ºC for 3 min; followed by 35 cycles of 94 ºC for 45 s, 65 ºC for 50 s and 60 s at 72 ºC; a final extension step at 72 ºC for 10 min was also performed.

DNA fragment "L" amplification was assembled with: 0.3 mM of a dNTPs mixture, 0.2 µM of each primer, 50 ng of DNA template, 1x DreamTaq buffer (containing optimized concentrations of KCl and $(NH_4)_2SO_4$ and 2 mM $MgCl_2$) and 2.5 U of DreamTaq DNA Polymerase (Thermo Scientific) in a total reaction volume of 50 µL. The amplification program executed was: 94 ºC for 3 min; followed by 35 cycles of 94 ºC for 45 s, 65 ºC for 50 s and 60 s at 72 ºC; a final extension step at 72 ºC for 10 min was also performed.

DNA fragment "Q" amplification was assembled with: 0.3 mM of a dNTPs mixture, 0.2 µM of each primer, 50 ng of DNA template, 1x DreamTaq buffer (containing optimized concentrations of KCl and $(NH_4)_2SO_4$ and 2 mM MgCl2) and 2.5 U of DreamTaq DNA Polymerase (Thermo Scientific) in a total reaction volume of 50 µL. The amplification program executed was: 94 ºC for 3 min; followed by 35 cycles of 94 ºC for 45 s, 65 ºC for 50 s and 60 s at 72 ºC; a final extension step at 72 ºC for 10 min was also performed.

DNA fragment "exon39" amplification was assembled with: 0.2 mM of a dNTPs mixture, 0.3 µM of each primer, 46 ng of DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA Polymerase (Invitrogen) in a total reaction volume of 50 µL. The amplification program used was: 94 ºC for 5 min; followed by 35 cycles of 94 ºC for 45 s, 60 ºC for 45 s and 60 s at 72 ºC; at the end the reaction was submitted to a final extension step at 72 ºC for 5 min.

DNA fragment "exon40" amplification was assembled with: 0.2 mM of a dNTPs mixture, 0.3 µM of each primer, 46 ng of DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA Polymerase (Invitrogen) in a total reaction volume of 50 µL. The amplification program used was: 94 ºC for 5 min; followed by 35 cycles of 94 ºC for 45 s, 60 ºC for 45 s and 60 s at 72 ºC; at the end the reaction was submitted to a final extension step at 72 ºC for 5 min.

DNA fragment "F" amplification was assembled with: 0.3 mM of a deoxynucleotide triphosphates (dNTPs) mixture, 0.2 µM of each primer, 59 ng of DNA

template, 1x DreamTaq buffer (containing optimized concentrations of KCl and $(NH_4)_2SO_4$ and 2 mM $MgCl_2$) and 2.5 U of DreamTaq DNA Polymerase (Thermo Scientific) in a total reaction volume of 50 µL. The amplification program executed was: 94 ºC for 2 min; followed by 35 cycles of 94 ºC for 30 s, 58 ºC for 50 s and 60 s at 72 ºC; a final extension step at 72 ºC for 10 min was also performed.

Further PCR products (for amplification of different gene fragments) were tested with different conditions and reagents, particularly: annealing temperature ranging 50-70 ºC; extension time ranging 60-360 s; different $MgCl_2$ concentrations; different primer and dNTPs concentrations and *Taq* polymerases (mainly DreamTaq DNA Polymerase (Thermo Scientific), *Taq* DNA Polymerase (Invitrogen), Taq DNA Polymerase (Thermo Scientific), Supreme NZYTaq DNA polymerase (NZYTech), NZYTaq DNA polymerase (NZYTech) and NZYTaq 2x Green Master Mix (NZYTech)).

Adding bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) in the PCR reaction mixtures was also tested. Moreover, primers of different sets were paired and tested to attempt amplification of longer products.

5. Electrophoresis and Purification of PCR products

In order to separate PCR products a 1% (w/v) agarose gel electrophoresis was performed. This universal technique can isolate different length DNA fragments, enabling a qualitative and quantitative overview of the amplified product when compared with a DNA ladder marker.

Tris-Borate-EDTA (TBE) 0.5X buffer (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.5) was used as electrophoresis buffer. PCR samples were loaded with 1X Orange DNA Loading Dye (100 ml: 400 mg Orange G, 40 mg sucrose) and 1X GelRed™ Nucleic Acid Gel Stain (Biotium). NZYDNA Ladder Marker III (NZYTech) was used as molecular marker. Gels ran for 1 - 2 h at 80 V and were visualized and photographed under UV light using a Kodak DC 120 camera (Sambrook et al., 1989).

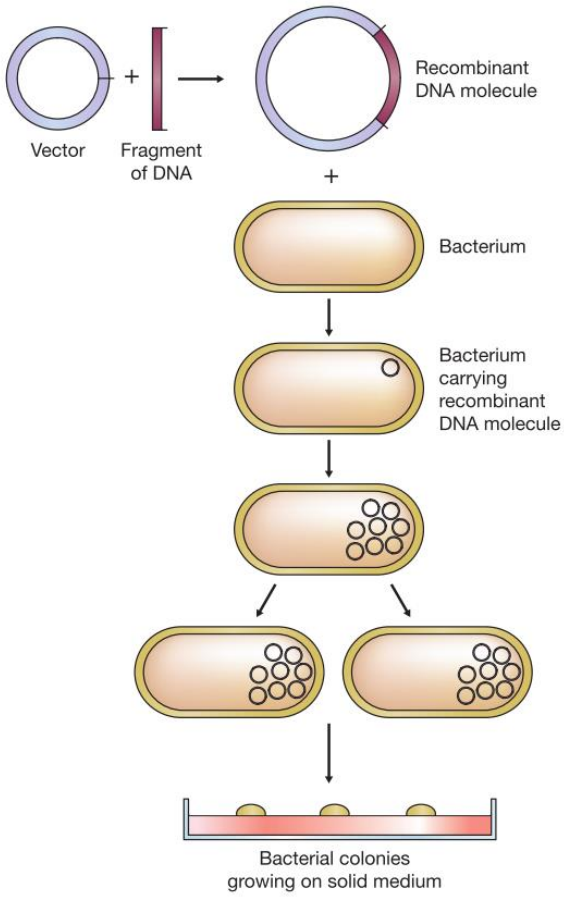
Expected product size bands were sliced out of the gel, when the product contained unspecific.

DNA purification and concentration was performed using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) for both direct purification from a PCR mixture or TBE agarose gel slice. The protocol is straightforward and includes the basic principles of sample capture, sample binding, wash and dry terminating with an elution step. Purified DNA was stored at -20 ºC until used.

An additional electrophoresis was performed after purification in order to confirm purified DNA integrity.

6. Cloning of PCR products

Modern genetic engineering techniques, particularly gene cloning, offer a rapid and efficient way to sequence target genes based on recombinant DNA technology. Gene cloning consists on the process of making copies or clones of a single target gene by assembling DNA recombinant molecules and replicating them in a host organism (see illustration 27).



Adapted from Brown (2010)

Illustration 27. The basic steps of gene cloning.

First, recombinant strands of DNA are to be made where plasmids are often used as vectors to transport the genes of interest to host cells.

Transformation is the process that comprises the transmission of the plasmid into a new host cell. This step requires that the host cells become "competent" in order to temporarily allow the entrance of extra DNA material; this can easily be achieved by a sudden change in environmental conditions such as temperature.

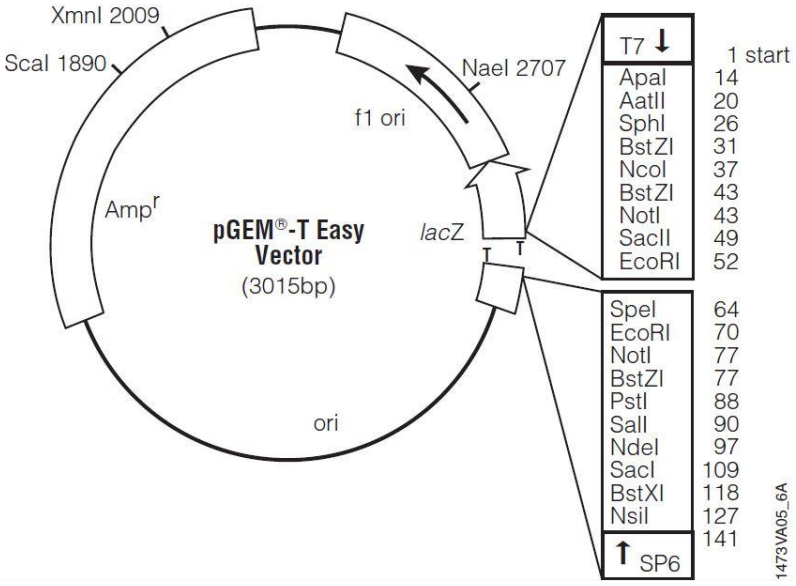
Escherichia coli (*E. coli*) are commonly used as hosts for DNA cloning and sequencing. Upon transformation of *E. coli* with a recombined vector carrying a gene of interest, the bacteria multiply the gene of interest while maintaining the integrity of its content.

Detection of transgenic cells is generally accomplished by expression of antibiotic resistance genes carried by the plasmids. Another common way of selection depends on the presence of proteins such as the X-Gal/ lacZ system.

6.1. Cloning vectors

6.1.1. pGem®-T Easy Vector cloning system

The pGEM®-T Easy Vector (Promega) is a high copy cloning vector with *ca.* 3.0 Kb of double stranded linearized DNA. This vector includes a gene for antibiotic resistance to ampicillin (Amp^r) and a gene for the enzyme β-galactosidase (*lacZ*) that contains the cloning site (illustration 28).



Adapted from pGEM®-T and pGEM®-T Easy Vector Systems

Illustration 28. pGEM®-T Easy Vector Map and points of interest, namely, Amp^r and lacZ gene locations.

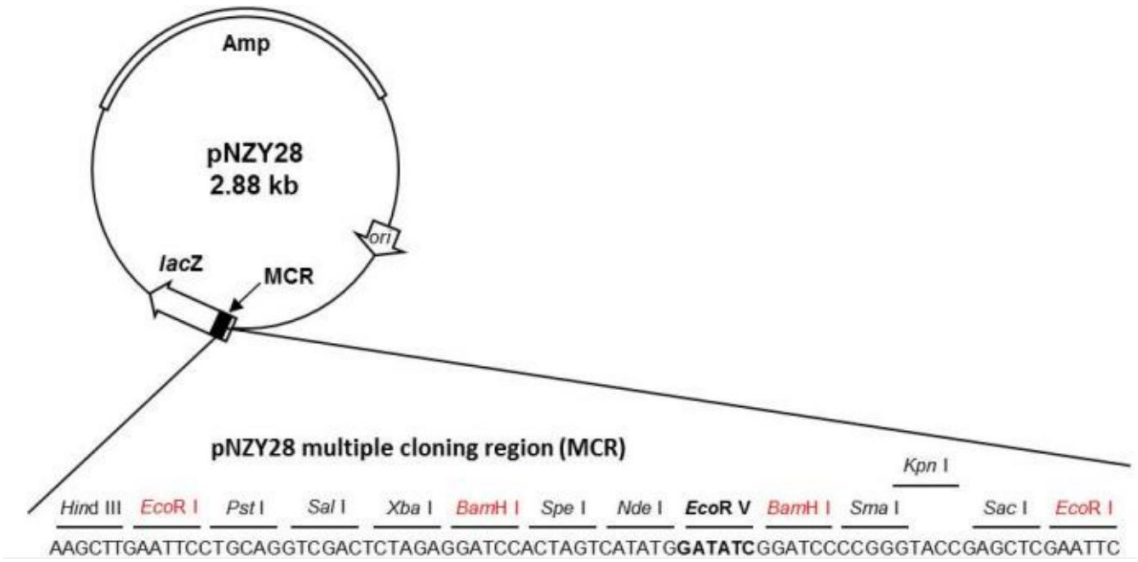
Cloning with this vector involves selection of transformants on ampicillin agar as well as screening for β-galactosidase activity to identify recombinants.

β-galactosidase is an enzyme that hydrolyzes a β-glycosidic bond as in lactose degradation producing galactose and glucose. In the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a lactose analog, the enzyme cleaves this specific bond inducing the production of galactose and 5-bromo-4-chloro-3-hydroxyindole. This compound is subsequently transformed into 5,5'-dibromo-4,4'-dichloro-indigo, via dimerization and oxidation, an insoluble blue product.

The expression of lacZ gene is induced by the lactose analog promoter IPTG (isopropyl-β-D-thiogalactopyranoside). The disruption of the lacZ gene by insertion of the desired DNA leads to white colonies defining lack of enzyme activity. On the other hand, blue colonies stand for non-recombinant bacteria that synthesized β-galactosidase and degraded X-Gal.

6.1.2. NZY-A PCR cloning kit

The pNZY28-A (NZYTech) is a high copy cloning vector with *ca.* 2.88 kb of double stranded linearized DNA that includes a gene for antibiotic resistance to ampicillin (Amp) and a lacZ gene. The cloning site is located in the lacZ gene for easy detection of recombinants by screening for β-galactosidase activity as with pGEM®-T Easy Vector (Promega) (illustration 29).



Adapted from NZY-A Speedy PCR cloning kit

Illustration 29. pNZY28 map and points of interest, namely, Amp and lacZ gene locations.

6.2. Plasmid-Insert Ligation

Ligation mixtures were prepared on sterile, nuclease-free microcentrifuge tubes with 5 µL of buffer, 50 ng of vector, 3 U of T4 DNA ligase and up to a ratio of 3:1 of insert (insert:vector ratio), in a 10 µL reaction that was incubated for 1 h 30 min at 25 ºC.

6.3. Transformation conditions

For *E.coli* transformation, JM109 competent cells (Promega) were used and manufacturer's instructions were followed. 5 µL of ligation reaction were added for each 50 µL of competent cells. The resultant mixture rested on ice for 20 min and was, then, subjected to a heat shock at 42 ºC for 45 s, before cooling for 2 min on ice. 950 µL of pre-warmed SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) were added to the mixture and the cells were left growing for 1 h 30 min at 37 ºC at 150 rpm on an orbital incubation shaker (Aerotron AG, HT Infors).

Cells were centrifuged at 1000 *g* for 10 min at room temperature and the resulting pellet was suspended in 70 µL of the supernatant, plated on low salt LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5, 1.5% agar) supplemented with 100 µg/mL of ampicillin, 0.5 mM IPTG and 80 µg/mL X-Gal, for selection of recombinant clones, and incubated overnight at 37 ºC.

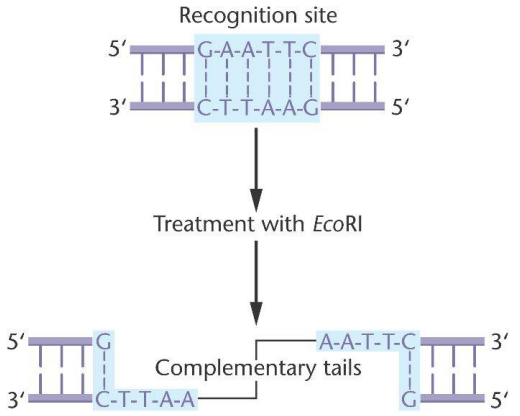
6.4. Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli* cells using GeneJET Plasmid Miniprep Kit (Thermo Scientific) following manufacturer's instructions, after growing cells in low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) supplemented with 100 µg/mL of ampicillin and grown overnight at 37 ºC at 150 rpm on an orbital incubation shaker (Aerotron AG, HT Infors). Extracted plasmid DNA was conserved at -20 ºC until use.

7. Restriction Assay

In order to confirm the selection of only proper recombinant clones, restriction assays were performed.

Restriction enzymes generally bind onto a specific recognition sequence of a DNA molecule, and subsequently cleave it by making an incision at the phosphate backbone of the double helix structure avoiding damages to the bases (illustration 30).



Adapted from Klug (2012)

Illustration 30. Operation of restriction enzymes – EcoRI.

FastDigest®*Eco*RI (Thermo Scientific) and FastDigest®NotI (Thermo Scientific) were the main restriction enzymes used. These endonucleases cut two specific regions of the plasmids, near the cloning site, allowing the linearization of the plasmid, plus the release of the cloned product (see points 6.1.1 and 6.1.2). Manufacturer's instructions were followed and results were viewed by agarose gel electrophoresis as described in point 5.

8. DNA Sequencing and Analysis

DNA sequencing reactions were performed by Macrogen (Netherlands). Sequence analysis was carried out using BioEdit Sequence Alignment Editor Version 7.1.7. and MEGA6. ClustalW Multiple Alignment, NCBI Basic Local Alignment Search Tool (BLAST) program and CAP contig assembly program were the main tools executed (Altschul et al., 1997; Hall, 1999).

9. Rapid Amplification of cDNA Ends

Previously described techniques bend to result in the acquisition of clones that represent only a part of the mRNA's complete sequence. Both missing sequence ends need a proper approach.

Rapid amplification of cDNA ends (RACE) is a powerful and inexpensive technique used to obtain the full-length sequence of an RNA transcript. In RACE technique, a short internal stretch of sequence must already be known from the mRNA. From this sequence, specific primers are chosen that are oriented in the direction of the missing sequence. Extension of the partial cDNAs from the unknown end back to the known region is achieved using primers that anneal to the preexisting poly(A) tail (3'-end) or to an appended homopolymer tail (5'-end) (Frohman et al., 1988).

9.1. 3’ RACE approach

Amplification of 3’ ends takes advantage of the poly(A) tail present in the messenger RNA. This chain of adenines enables amplification by PCR using an oligo(dT) anchor primer, fully constituted of thymines and an internal primer from the nearest known location (a reverse transcription primer can be used) (illustration 31).

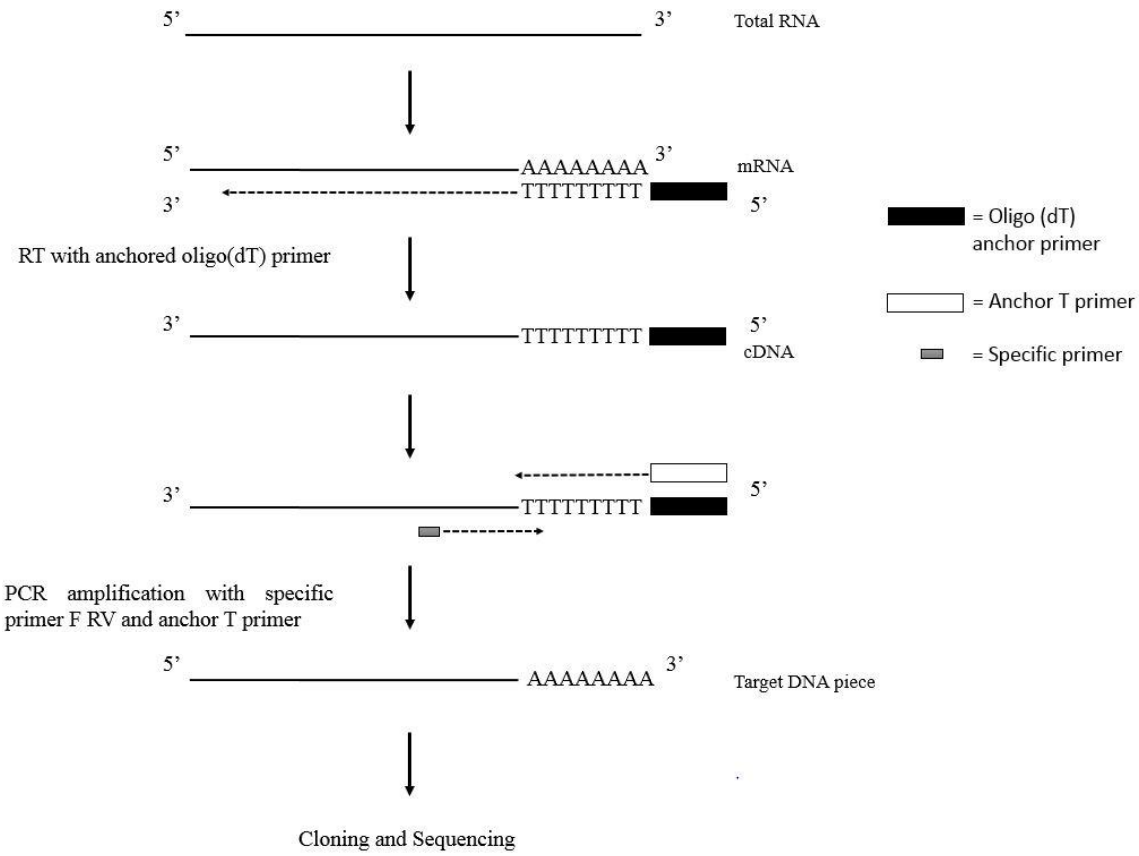


Illustration 31. Summary of the FASN 3’ end amplification by RACE.

First, a RT reaction was performed with the extracted total RNA. Up to 3 µg of total RNA were converted in a final 20 µL reaction with 60 mM oligo d(T) anchor primer (table 3). Initial denaturation was achieved by a 5 min incubation at 70 ºC followed by 10 min on ice. Then, reverse transcription was performed in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs and with 40 U RNase Out (Invitrogen) and 200 U of M-MLV reverse transcriptase (Invitrogen), for 1 h at 37 ºC, followed by 10 min at 70 ºC for enzyme denaturation.

Table 3. List of primers used for FASN 3' end sequencing.

Primer Designation	**Gene Location (bp)**	**5'→ 3' Sequence**
FAS F RV	7490-7511	TGTGGATGATGCTGAGGATGGA
FAS RACE 3'	7452-7477	CACCGCACGCTGCTGGAGGGC
Oligo d(T) anchor NotI	-	AACCCGGCTCGAGCGGCCGCT_{18}
Anchor T	-	AACCCGGCTCGAGCGGCCGC

For amplification of the FASN 3' end region a PCR assay was designed with the following conditions: 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, 0.1 mg BSA, 2 mM $MgSO_4$, 0.2 µM of FAS F RV and Anchor T and 2.5 U of *Pfu* DNA Polymerase (Thermo Scientific) in a total reaction volume of 50 µL. The amplification program was: 94 ºC for 2 min; 10 cycles of 94 ºC for 15 s followed by 55 ºC for 30 s and an extension time of 40 s at 72 ºC; 94 ºC for 15 s; 25 cycles of 94 ºC for 15 s followed by 55 ºC for 30 s and an extension stage of 72 ºC for 40 s with an increment of 20 s each cycle; 72 ºC for 7 min. The product was analyzed in a 1% agarose gel that ran for 1 h 30 min at 70 V at constant voltage. Samples were prepared according to what is described in point 5. Expected product size bands were sliced and purified with illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturer's instructions. Target DNA was cloned with pGEM®-T Easy Vector (Promega) and multiplied in an *E. coli* host as described in point 6. Multiple clones were obtained and sequenced as described in points 6, 7 and 8. However, none of the sequenced clones proved to be a successfully amplified FASN product. Furthermore, another attempt to sequence the FASN 3' end was made using another primer named "FAS RACE 3'"(Table 3), using the same conditions.

IV. RESULTS

1. Fragment amplification detection

PCR amplification produced expected amplicons of the gene that were analyzed by agarose electrophoresis gels (illustration 32). Designated specific primers, originated previously calculated amplified products of about 430, 380 and 780 bp, respectively.

PCR products were cloned in pNZY28 (NZYTech) or pGEM®-T Easy Vector (Promega). Multiple clones of each were obtained and sequenced.

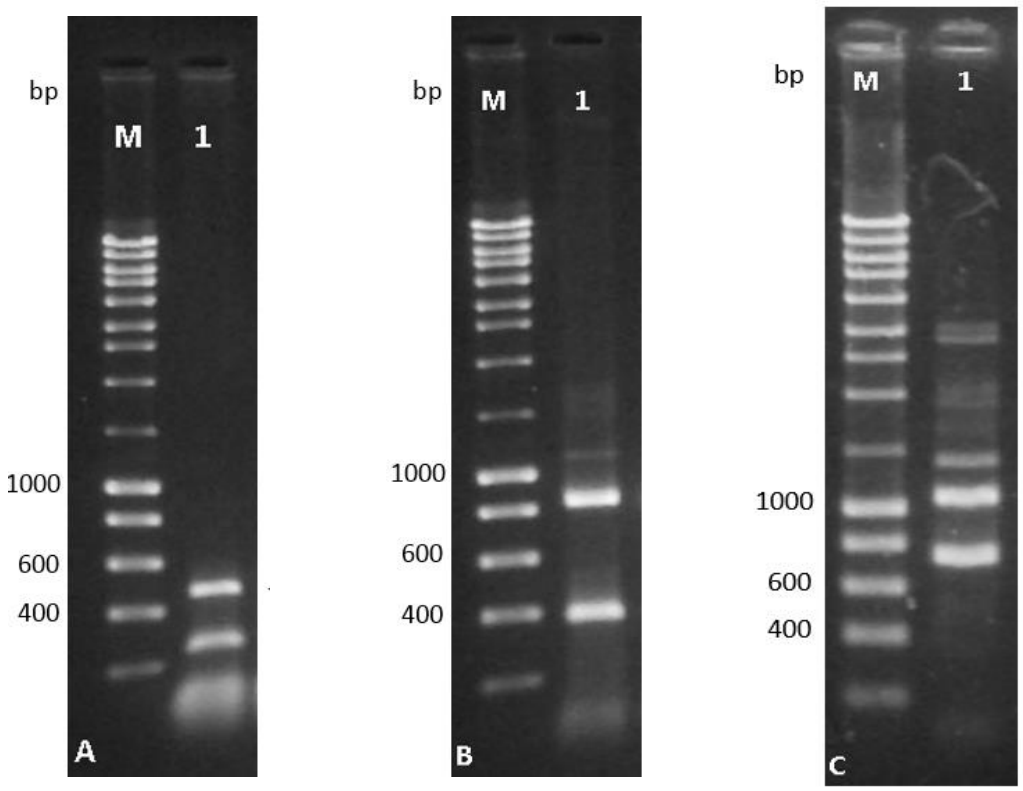


Illustration 32. Examples of amplicons generated in PCR assays using distinct primers observed by 1% agarose gel electrophoresis. **A**: Lane M – NZYDNA Ladder Marker III (NZYTech), Lane 1 – PSUBexon39; **B**: Lane M – NZYDNA Ladder Marker III (NZYTech), Lane 1 – PSUBexon40; **C**: Lane M – NZYDNA Ladder Marker III (NZYTech), Lane 1– PSUBL.

2. Detection of the product after restriction assay

Restriction assays were performed in order to confirm the authenticity of the clones before sequencing. FastDigest®EcoRI (Thermo Scientific) and FastDigest®NotI (Thermo Scientific) were the main restriction enzymes used to digest the plasmid and release the cloned product. Results were observed by electrophoresis of 1% agarose gels (illustration 33).

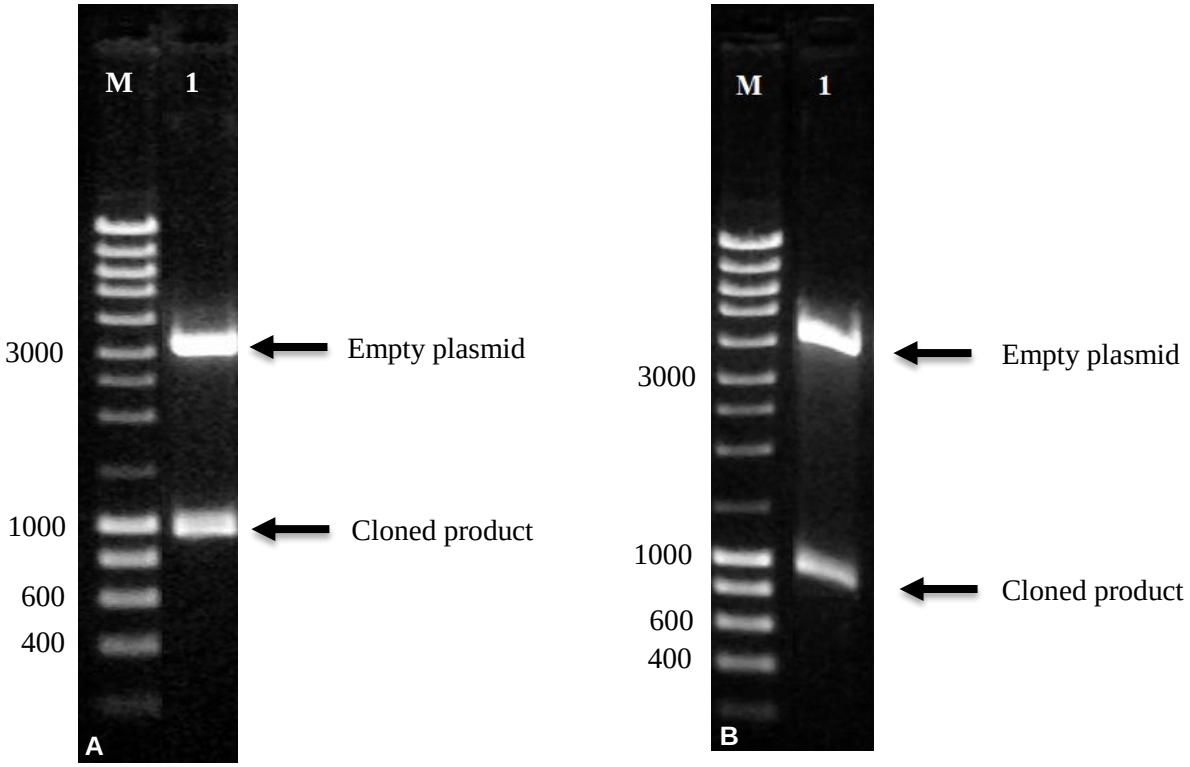
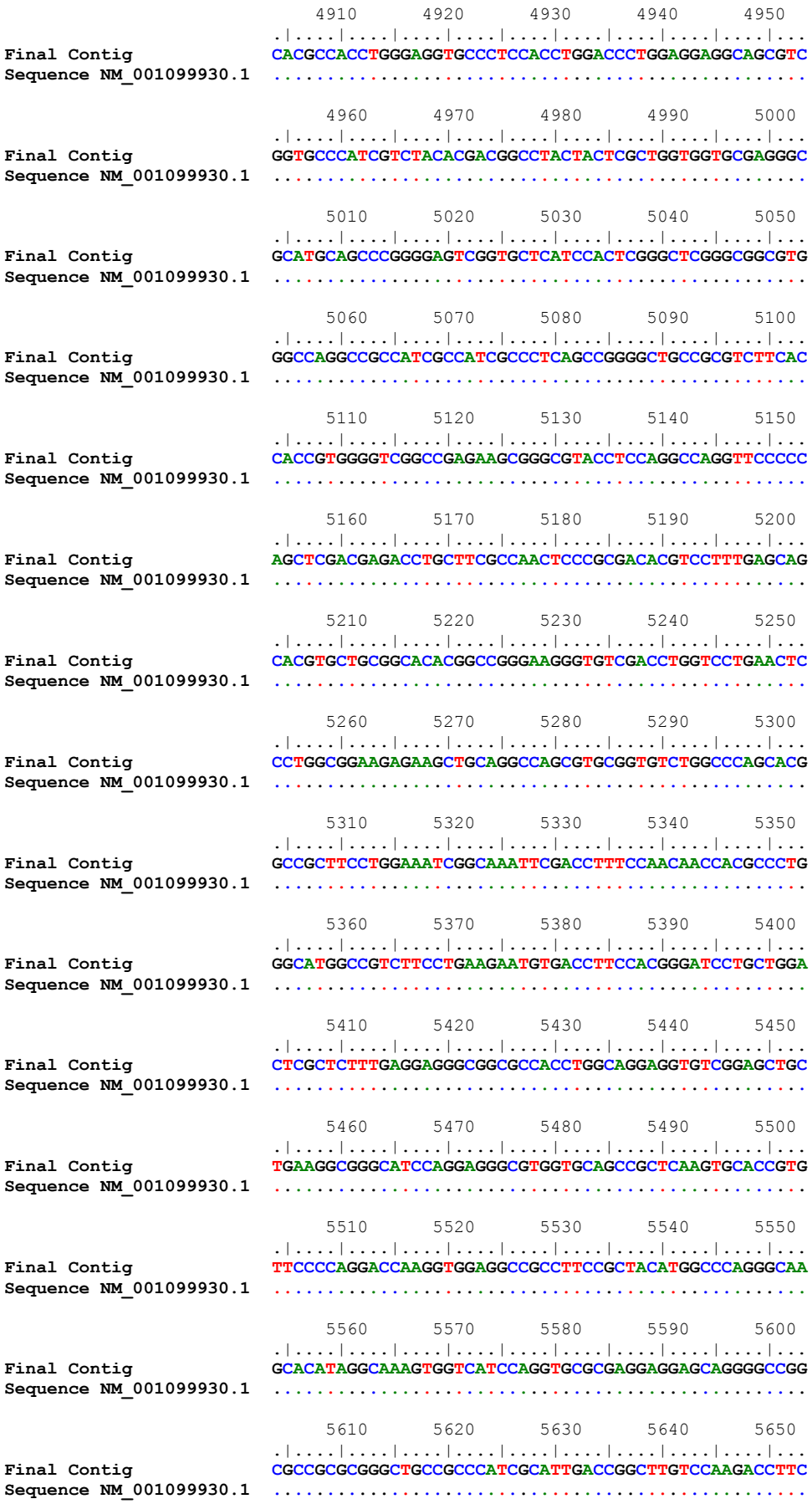


Illustration 33. Example of an insert release gel after the restriction assay. A: Lane M - NZYDNA Ladder Marker III (NZYTech); Lane 1 – PSUBL. B: Lane M – NZYDNA Ladder Marker III (NZYTech); Lane 1 – PSUBQ.

3. Molecular characterization of the sequenced results

Assembly of the final contig from the overlapping of the sequenced DNA segments was achieved using BioEdit and MEGA software. This sequence was compared to an available 8044 bp swine FASN sequence with the reference number NM_001099930.1 present on NCBI database and covered from 4903-7489 bp (illustration 34). The complete sequence of each successfully sequenced amplicon can be found in appendix II.


4910 4920 4930 4940 4950
Final Contig CACGCCACCTGGGAGGTGCCCTCCACCTGGACCCTGGAGGAGGCAGCGTC
Sequence NM_001099930.1 .....
4960 4970 4980 4990 5000
Final Contig GGTGCCCATCGTCTACACGACGGCCTACTACTCGCTGGTGGTGCGAGGGC
Sequence NM_001099930.1 .....
5010 5020 5030 5040 5050
Final Contig GCATGCAGCCCGGGGAGTCGGTGCTCATCCACTCGGGCTCGGGCGGCGTG
Sequence NM_001099930.1 .....
5060 5070 5080 5090 5100
Final Contig GGCCAGGCCGCCATCGCCATCGCCCTCAGCCGGGGCTGCCGCGTCTTCAC
Sequence NM_001099930.1 .....
5110 5120 5130 5140 5150
Final Contig CACCGTGGGGTCGGCCGAGAAGCGGGCGTACCTCCAGGCCAGGTTCCCCC
Sequence NM_001099930.1 .....
5160 5170 5180 5190 5200
Final Contig AGCTCGACGAGACCTGCTTCGCCAACTCCCGCGACACGTCCTTTGAGCAG
Sequence NM_001099930.1 .....
5210 5220 5230 5240 5250
Final Contig CACGTGCTGCGGCACACGGCCGGGAAGGGTGTCGACCTGGTCCTGAACTC
Sequence NM_001099930.1 .....
5260 5270 5280 5290 5300
Final Contig CCTGGCGGAAGAGAAGCTGCAGGCCAGCGTGCGGTGTCTGGCCCAGCACG
Sequence NM_001099930.1 .....
5310 5320 5330 5340 5350
Final Contig GCCGCTTCCTGGAAATCGGCAAATTCGACCTTTCCAACAACCACGCCCTG
Sequence NM_001099930.1 .....
5360 5370 5380 5390 5400
Final Contig GGCATGGCCGTCTTCCTGAAGAATGTGACCTTCCACGGGATCCTGCTGGA
Sequence NM_001099930.1 .....
5410 5420 5430 5440 5450
Final Contig CTCGCTCTTTGAGGAGGGCGGCGCCACCTGGCAGGAGGTGTCGGAGCTGC
Sequence NM_001099930.1 .....
5460 5470 5480 5490 5500
Final Contig TGAAGGCGGGCATCCAGGAGGGCGTGGTGCAGCCGCTCAAGTGCACCGTG
Sequence NM_001099930.1 .....
5510 5520 5530 5540 5550
Final Contig TTCCCCAGGACCAAGGTGGAGGCCGCCTTCCGCTACATGGCCCAGGGCAA
Sequence NM_001099930.1 .....
5560 5570 5580 5590 5600
Final Contig GCACATAGGCAAAGTGGTCATCCAGGTGCGCGAGGAGGAGCAGGGGCCGG
Sequence NM_001099930.1 .....
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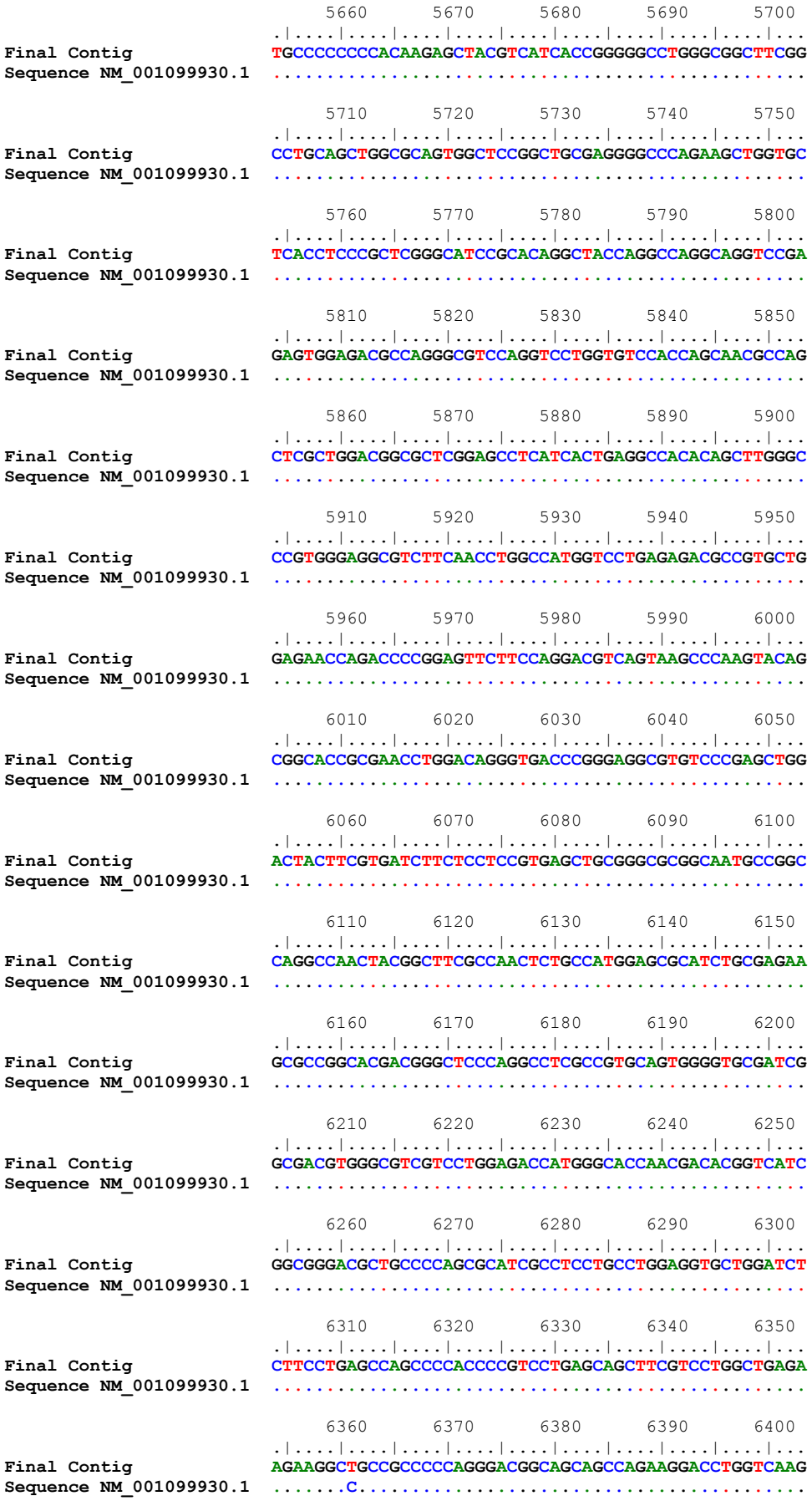
5660 5670 5680 5690 5700
Final Contig TGCCCCCCCCACAAGAGCTACGTCATCACCGGGGGCCTGGGCGGCTTCGG
Sequence NM_001099930.1 .....
5710 5720 5730 5740 5750
Final Contig CCTGCAGCTGGCGCAGTGGCTCCGGCTGCGAGGGGCCCAGAAGCTGGTGC
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5760 5770 5780 5790 5800
Final Contig TCACCTCCCGCTCGGGCATCCGCACAGGCTACCAGGCCAGGCAGGTCCGA
Sequence NM_001099930.1 .....
5810 5820 5830 5840 5850
Final Contig GAGTGGAGACGCCAGGGCGTCCAGGTCCTGGTGTCCACCAGCAACGCCAG
Sequence NM_001099930.1 .....
5860 5870 5880 5890 5900
Final Contig CTCGCTGGACGGCGCTCGGAGCCTCATCACTGAGGCCACACAGCTTGGGC
Sequence NM_001099930.1 .....
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Final Contig CCGTGGGAGGCGTCTTCAACCTGGCCATGGTCCTGAGAGACGCCGTGCTG
Sequence NM_001099930.1 .....
5960 5970 5980 5990 6000
Final Contig GAGAACCAGACCCCGGAGTTCTTCCAGGACGTCAGTAAGCCCAAGTACAG
Sequence NM_001099930.1 .....
6010 6020 6030 6040 6050
Final Contig CGGCACCGCGAACCTGGACAGGGTGACCCGGGAGGCGTGTCCCGAGCTGG
Sequence NM_001099930.1 .....
6060 6070 6080 6090 6100
Final Contig ACTACTTCGTGATCTTCTCCTCCGTGAGCTGCGGGCGCGGCAATGCCGGC
Sequence NM_001099930.1 .....
6110 6120 6130 6140 6150
Final Contig CAGGCCAACTACGGCTTCGCCAACTCTGCCATGGAGCGCATCTGCGAGAA
Sequence NM_001099930.1 .....
6160 6170 6180 6190 6200
Final Contig GCGCCGGCACGACGGGCTCCCAGGCCTCGCCGTGCAGTGGGGTGCGATCG
Sequence NM_001099930.1 .....
6210 6220 6230 6240 6250
Final Contig GCGACGTGGGCGTCGTCCTGGAGACCATGGGCACCAACGACACGGTCATC
Sequence NM_001099930.1 .....
6260 6270 6280 6290 6300
Final Contig GGCGGGACGCTGCCCCAGCGCATCGCCTCCTGCCTGGAGGTGCTGGATCT
Sequence NM_001099930.1 .....
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Final Contig CTTCCTGAGCCAGCCCCACCCCGTCCTGAGCAGCTTCGTCCTGGCTGAGA
Sequence NM_001099930.1 .....
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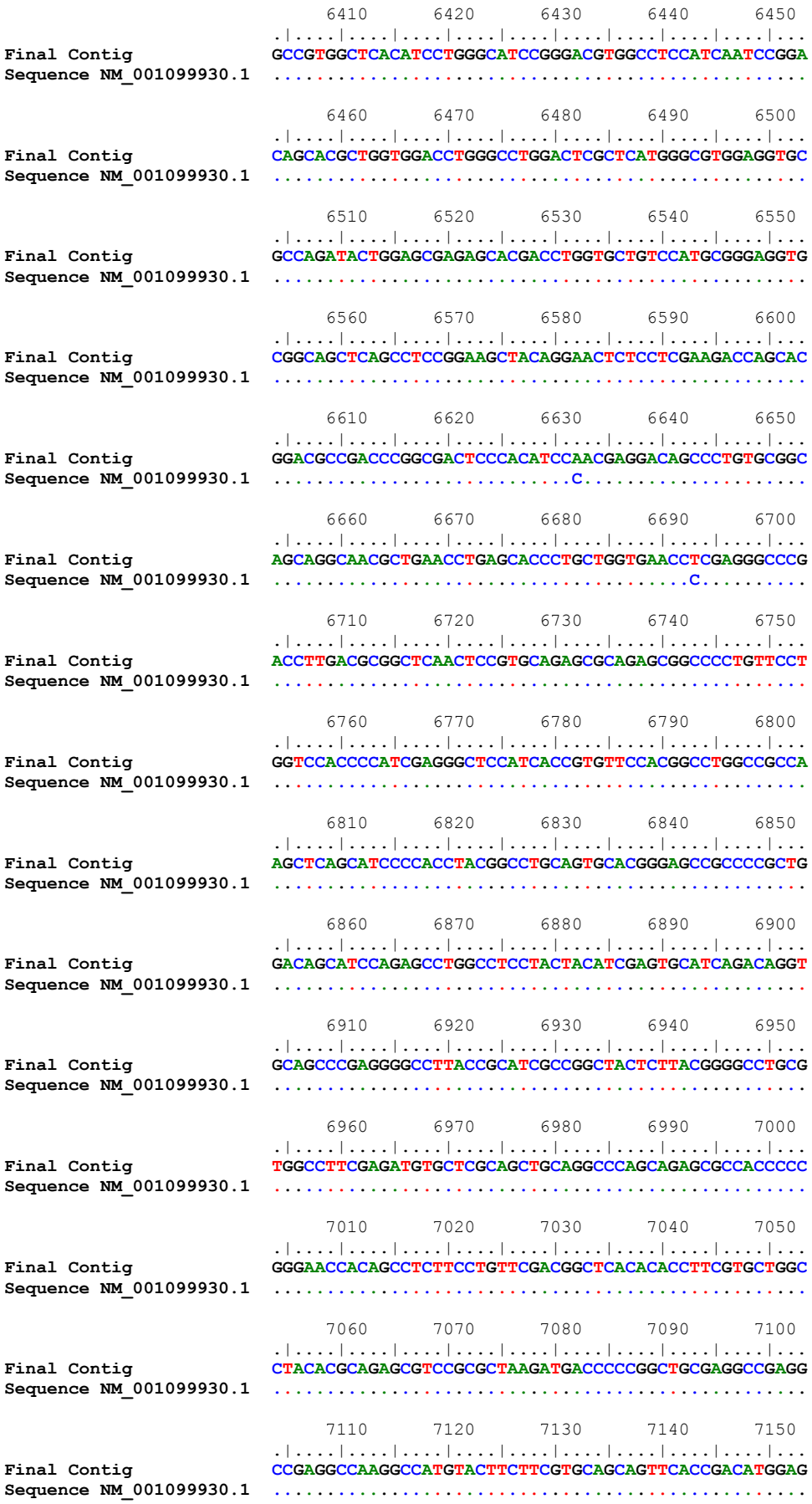
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Sequence NM_001099930.1 .....
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Final Contig GCCAGATACTGGAGCGAGAGCACGACCTGGTGCTGTCCATGCGGGAGGTG
Sequence NM_001099930.1 .....
6560 6570 6580 6590 6600
Final Contig CGGCAGCTCAGCCTCCGGAAGCTACAGGAACTCTCCTCGAAGACCAGCAC
Sequence NM_001099930.1 .....
6610 6620 6630 6640 6650
Final Contig GGACGCCGACCCGGCGACTCCCACATCCAACGAGGACAGCCCTGTGCGGC
Sequence NM_001099930.1 .....C.....
6660 6670 6680 6690 6700
Final Contig AGCAGGCAACGCTGAACCTGAGCACCCTGCTGGTGAACCTCGAGGGCCCG
Sequence NM_001099930.1 .....C.....
6710 6720 6730 6740 6750
Final Contig ACCTTGACGCGGCTCAACTCCGTGCAGAGCGCAGAGCGGCCCCTGTTCCT
Sequence NM_001099930.1 .....
6760 6770 6780 6790 6800
Final Contig GGTCCACCCCATCGAGGGCTCCATCACCGTGTTCCACGGCCTGGCCGCCA
Sequence NM_001099930.1 .....
6810 6820 6830 6840 6850
Final Contig AGCTCAGCATCCCCACCTACGGCCTGCAGTGCACGGGAGCCGCCCCGCTG
Sequence NM_001099930.1 .....
6860 6870 6880 6890 6900
Final Contig GACAGCATCCAGAGCCTGGCCTCCTACTACATCGAGTGCATCAGACAGGT
Sequence NM_001099930.1 .....
6910 6920 6930 6940 6950
Final Contig GCAGCCCGAGGGGCCTTACCGCATCGCCGGCTACTCTTACGGGGCCTGCG
Sequence NM_001099930.1 .....
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Final Contig TGGCCTTCGAGATGTGCTCGCAGCTGCAGGCCCAGCAGAGCGCCACCCCC
Sequence NM_001099930.1 .....
7010 7020 7030 7040 7050
Final Contig GGGAACCACAGCCTCTTCCTGTTCGACGGCTCACACACCTTCGTGCTGGC
Sequence NM_001099930.1 .....
7060 7070 7080 7090 7100
Final Contig CTACACGCAGAGCGTCCGCGCTAAGATGACCCCCGGCTGCGAGGCCGAGG
Sequence NM_001099930.1 .....
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Final Contig CCGAGGCCAAGGCCATGTACTTCTTCGTGCAGCAGTTCACCGACATGGAG
Sequence NM_001099930.1 .....

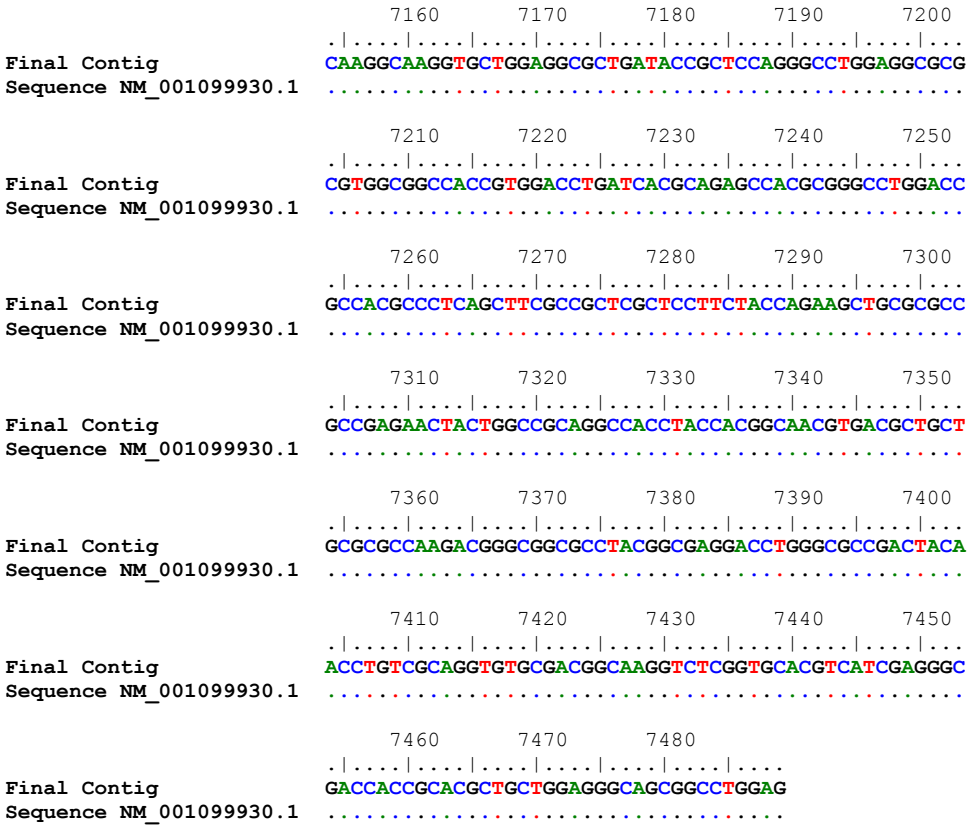
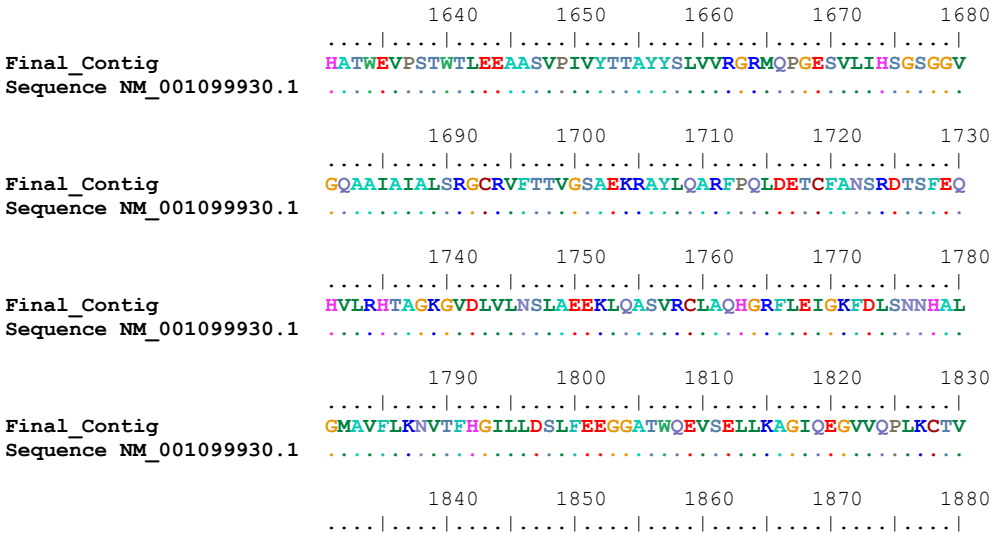


Illustration 34. Nucleotide sequence alignment of total sequenced product with swine FASN sequence NM_001099930.1.

The three previously displayed nucleotide differences reflect in two changes of amino acids in the translated sequence (an asparagine for a histidine and a leucine for a proline) (illustration 35).



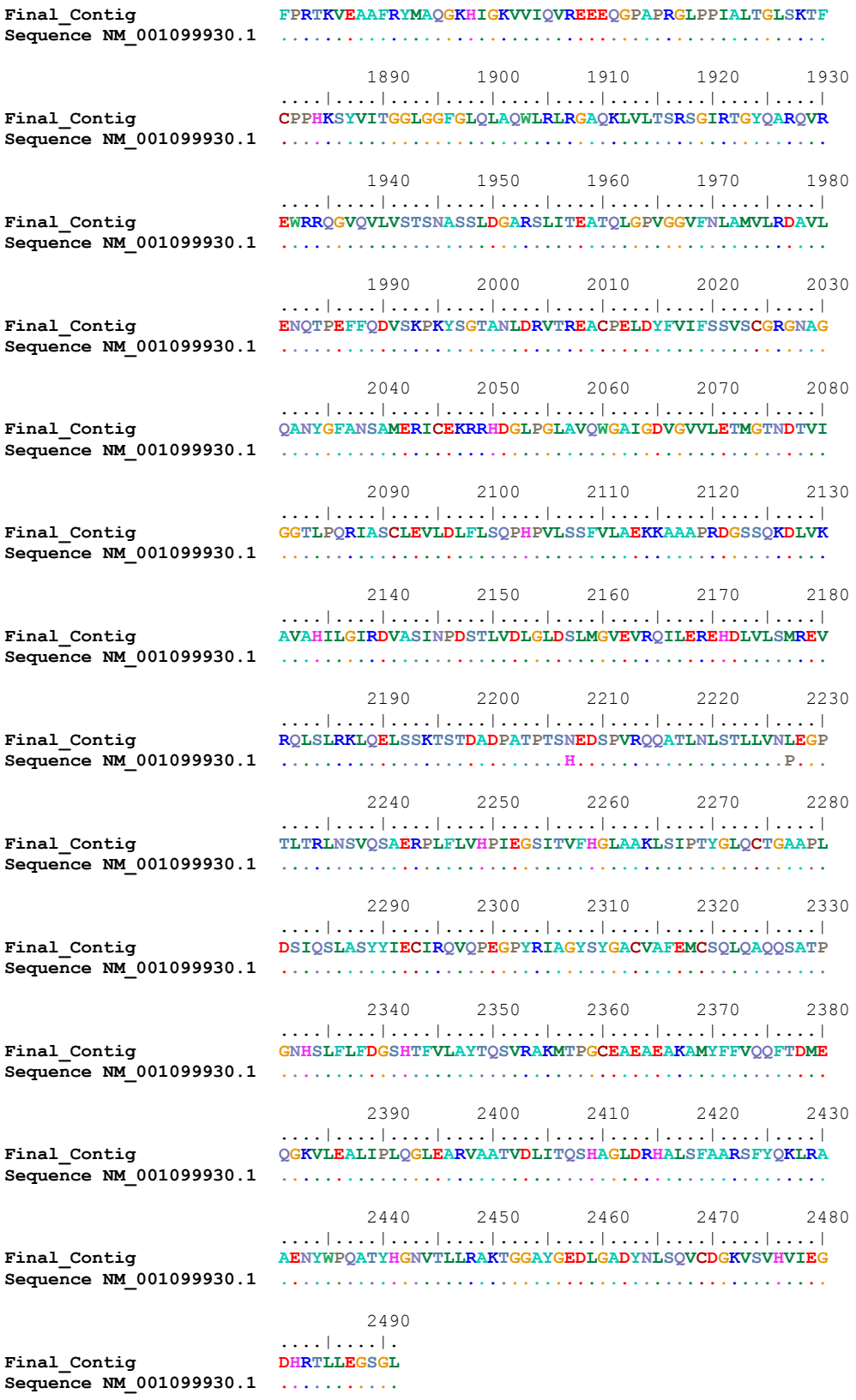


Illustration 35. Protein sequence alignment of total sequenced product with swine FASN sequence NM_001099930.1.

V. DISCUSSION

Knowledge on the genetic background of fat tissue accumulation is of great importance in livestock production and as well as in biomedical research.

No references were found in current literature about *Sus mediterraneus* FASN specific primer sequences. Meanwhile, from the five pairs *Sus scrofa* FASN specific primer sequences previously described and fully tested in this work, only the product obtained from the use of two pairs (FASN39-FW/FASN39-RV and FASN40-FW/FASN40-RV) (Kim et al., 2011) have been successfully amplified and sequenced. The other ones, although submitted to different conditions (see 4.3 from chapter III) haven't given products successfully amplified and/or sequenced. Therefore, this work is based on the amplicons obtained with the 2 pairs of primers above mentioned and with other 4 pairs, from the 9 pairs designed and tested by the author.

Several FASN polymorphisms described in literature in cattle have been found associated with meat FA composition (Bhuiyan et al., 2009; Oztabak et al., 2014; Zhang et al., 2008). Furthermore, some of them have been assigned to the KR and TE domains. In humans, some reported FASN SNPs have been related with higher BMI, increased risk for developing prostate cancer and associated with dietary fat intakes (Bouchard-Mercier et al., 2012; Nguyen et al., 2010).

In swine FASN, some SNPs have already been described in breeds other than the AL/Iberian pig, most of them related to meat quality and FA composition (Kim et al., 2011; Munoz et al., 2007).

Our results on a partial sequence of the AL fatty acid synthase gene have shown three main exonic polymorphisms, two of them missense and one silent/synonymous (table 4).

Table 4. Characterization of the identified polymorphisms. Nomenclature of mutations follows Ogino et al. (2007)

DNA sequence change	Type of mutation	Amino acid change (three-letter code)	FASN site of mutation (exon)
c.6361C>T	synonymous	-	38
c.6632C>A	Non-synonymous	p.His2207Asn	39
c.6693C>T	Non-synonymous	p.Pro2227Leu	40

At the nucleotide position 6361, a cytosine was substituted by a thymine with no further consequences on the translated triplet (alanine, GCC > GCT). As this mutation does not conduce to an amino acid change, it is designated by synonymous single nucleotide polymorphism (sSNP).

At the nucleotide position 6632, a cytosine was substituted by an adenine, conducing to an alteration on the amino acid residue synthesized, from histidine (CAC) to asparagine (AAC) which is therefore named a non-synonymous single nucleotide polymorphism (nsSNP).

Finally, at the nucleotide position 6693, a cytosine was substituted by a thymine conducing to an nsSNP, because it modifies the translated sequence, a proline (CCC) for a leucine (CTC).

The structure of ACP and TE domains are not yet entirely resolved in the porcine FASN (Maier, 2008). Both nsSNPs identified in this work are located in this non determined coding region of the AL FASN gene. Nevertheless, c.6632C>A (His-Asn) is a polymorphism that has already been identified in other studies with *Sus scrofa* (accession number XP_003482986.2) although the origin of the biological material is not mentioned (tissue type and animal breed) and it has no publication associated to it.

Similarity coefficients between the sequenced AL FASN and the database record NM_001099930.1 (*Sus scrofa*) confirm that they are almost identical whether in terms of nucleotides (99,99%) or at the protein level (99,99%).

The AL FASN exonic sequence presented in this study covers part of the ER domain (4904 - 5575), part the KR domain (<5639 - 6349) and part of the TE domain (6740 - 7489). Additionally, this sequence covers the coding region of the phosphopantetheine attachment site (6359 - >6553), the short chain dehydrogenase region (5714 - 6172), several putative NADP binding sites, active sites and several NAD(P) binding sites. These regions have found to be highly conservative between AL and *Sus scrofa*. Estimate locations of the different regions were obtained from the reference sequence NM_001099930.1.

Sequence data obtained with this work were already deposited on the NCBI GenBank database under the accession number KM658506.

The KR domain is responsible for the first reduction in the FA synthesis cycle. In the presence of NADPH, it catalyzes the reduction of acetoacetyl ACP to D-3-hydroxybutyryl ACP. After dehydration, the ER domain catalyzes the second reduction step of the acyl intermediate trans-Δ2-enoyl ACP into butyryl ACP, again with NADPH as electron donor (Berg et al., 2012).

During FA synthesis, the growing acyl intermediate is attached to an ACP throughout the elongation cycles until a 16 carbon chain lenght FA is released by a TE. This domain leads to the release of palmitate by the hydrolysis of the acyl-S-

phosphopantetheine thioester bound to the preceding ACP domain (Chakravarty et al., 2004).

TE domains are important regions in the FASN gene because they are potential substrates for elongation and denaturation that are responsible for length determination of the FASN gene. The structure of the human TE domain is already determined. It is comprised of two dissimilar subdomains, A and B. The structure has a hydrophobic groove with a distal pocket at the interface of the two subdomains, constituting the candidate substrate binding site. As the TE domain ensures that the majority of FAS product is palmitate (C16), variations within this domain coding sequence may influence the structure of substrate-binding site and affect the specific activity towards acyl ACP intermediates (Chakravarty et al., 2004; Maharani et al., 2011; Zhang et al., 2008).

Moreover, Zhang et al. (2008) identified, in bovines, two novel nonsynonymous mutations in the TE domain of FAS, g.18663T>C and g.18727C>T, which were associated with decreased amounts of unsaturated fatty acids. These TE polymorphisms might be related to the ones described hereby, though complementary studies are required to analyze such hypothesis.

ACP is a single polypeptide constituted of 77 residues with scarce publication related to it about polymorphisms among different species (Berg et al., 2012).

A recent genome study on Mangalica (Molnar et al., 2014), an obese local pig of Hungary, concluded that local/rare breeds could be a rich source of sequence variations not present in industrial/lean breeds. Therefore, identified variations within such breeds, as the AL, might be useful resource for future studies of pig’s traits.

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Three polymorphisms were identified in about 33% of the total gene sequence. Two of these SNP's, namely c.6632C>A and c.6693C>T, lead to changes in the translated triplet (missense mutations) and are located somewhere around the acyl carrier protein and thioesterase FASN domains, locations not yet fully understood in swine, particularly in terms of structure. This fact suggests that further complementary studies are required in order to investigate possible consequences induced by these polymorphisms and relate them with previously described ones on other swine breeds and/or species.

On the other hand, it is necessary to complete the genotyping of the FASN in the AL breed since the discovery of more FASN polymorphisms on the remaining coding sequence that may provide useful information, is a solid possibility.

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APPENDIX I – COMPLETE LIST OF TESTED PRIMERS

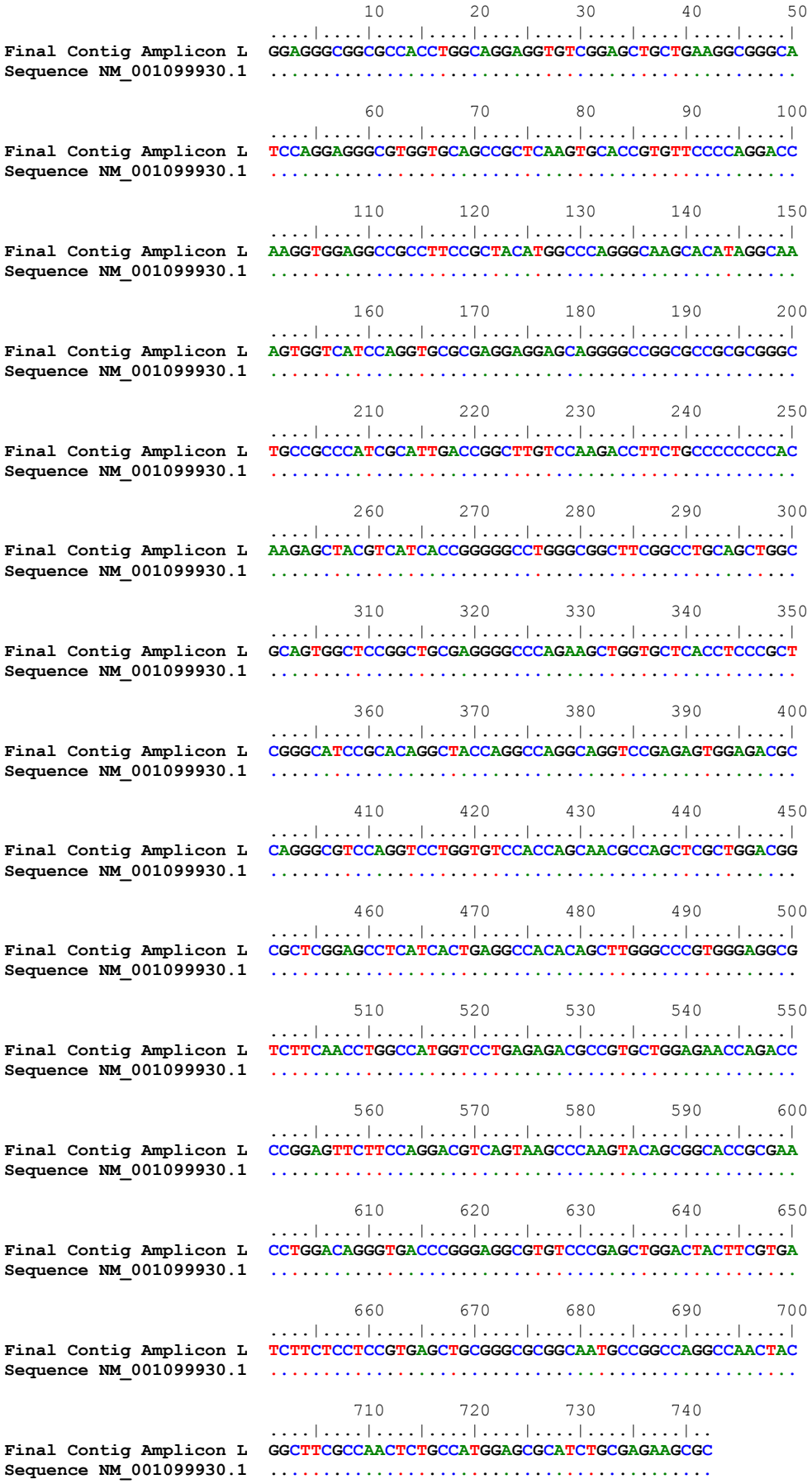
Primer Designation	Gene Location (bp)	5'→ 3' Primer Sequence	Sense/ Antisense
FAS S	119-139	GACGACAGGCGGTGGAAGGCG	Sense
FAS AS	749-771	GTGTTCGTGCCCGCATTGAGGAT	Antisense
FAS T FW	1216-1236	CGTCATCCTTCAGCCCAACTC	Sense
FAS T RV	2578-2599	ATAGACGGCGACAGAGGAGCAG	Antisense
FAS P FW	2358-2375	TCCCGCTGATGAAGAAGG	Sense
FAS P RV	4084-4101	GTGAGGAAGCCGACCATC	Antisense
FAS Y FW	3735-3754	AGATGAAGGTGGTGGAGGTG	Sense
FAS Y RV	5549-5568	GGCAAGCACATAGGCAAAGT	Antisense
FAS X FW	5463-5479	GCATCCAGGAGGGCGTG	Sense
FAS X RV	6615-6631	GGATGTGGGAGTCGCCG	Antisense
FASN exon39-FW	6615-6631	CGGCGACTCCCACATCC	Sense
FASN exon39-RV	7025-7045	GAAGGTGTGTGAGCCGTCGAA	Antisense
FASN exon40-FW	6908-6928	CCCGAGGGGCCTTACCGCATC	Sense
FASN exon40-RV	7268-7285	GAAGGAGCGAGCGGCGAA	Antisense
FAS F FW	7015-7035	CCTCTTCCTGTTCGACGGCTC	Sense
FAS F RV	7490-7511	TGTGGATGATGCTGAGGATGGA	Antisense
FASN1 FW	88-115	CCTCATCGGCGGTGTGGA	Sense
FASN1 RV	442-465	CTGAAGTCGAAGAAGAAGGAGAGC	Antisense
FASN2 FW	435-455	CCAACCGGCTCTCCTTCTTCT	Sense
FASN2 RV	873-894	CCGTGGGCTTCGATGTATTCAA	Antisense
FAS L FW	5393-5415	ATCCTGCTGGACTCGCTCTTTGA	Sense
FAS L RV	6158-6175	TGGGAGCCCGTCGTGCCG	Antisense
FAS Q FW	6108-6130	CCAACTACGGCTTCGCCAACTCT	Sense
FAS Q RV	6746-6767	CGATGGGGTGGACCAGGAACAG	Antisense
FAS D FW	4881-4902	CCACCTCTGTTCTGCTGCTTCA	Sense
FAS D RV	5546-5565	TTGCCTATGTGCTTGCCCTG	Antisense
FAS H FW	4092-4114	GCTTCCTCACCTCCCCTGAACAA	Sense
FAS H RV	5020-5040	CCCGAGTGGATGAGCACCGAC	Antisense
FAS RACE 3'	7452-7477	CACCGCACGCTGCTGGAGGGC	Sense
Oligo d(T) anchor NotI	-	AACCCGGCTCGAGCGGCCGCT$_{18}$	-
Anchor T	-	AACCCGGCTCGAGCGGCCGC	-

APPENDIX II – FULL ALIGNMENT PRESENTATION OF EACH SEQUENCED DNA PORTION

Amplicon D:

```
                                10        20        30        40        50
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  CACGCCACCTGGGAGGTGCCCTCCACCTGGACCCTGGAGGAGGCAGCGTC
Sequence NM_001099930.1  ..................................................

                                60        70        80        90       100
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  GGTGCCCATCGTCTACACGACGGCCTACTACTCGCTGGTGGTGCGAGGGC
Sequence NM_001099930.1  ..................................................

                               110       120       130       140       150
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  GCATGCAGCCCGGGGAGTCGGTGCTCATCCACTCGGGCTCGGGCGGCGTG
Sequence NM_001099930.1  ..................................................

                               160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  GGCCAGGCCGCCATCGCCATCGCCCTCAGCCGGGGCTGCCGCGTCTTCAC
Sequence NM_001099930.1  ..................................................

                               210       220       230       240       250
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  CACCGTGGGGTCGGCCGAGAAGCGGGCGTACCTCCAGGCCAGGTTCCCCC
Sequence NM_001099930.1  ..................................................

                               260       270       280       290       300
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  AGCTCGACGAGACCTGCTTCGCCAACTCCCGCGACACGTCCTTTGAGCAG
Sequence NM_001099930.1  ..................................................

                               310       320       330       340       350
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  CACGTGCTGCGGCACACGGCCGGGAAGGGTGTCGACCTGGTCCTGAACTC
Sequence NM_001099930.1  ..................................................

                               360       370       380       390       400
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  CCTGGCGGAAGAGAAGCTGCAGGCCAGCGTGCGGTGTCTGGCCCAGCACG
Sequence NM_001099930.1  ..................................................

                               410       420       430       440       450
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  GCCGCTTCCTGGAAATCGGCAAATTCGACCTTTCCAACAACCACGCCCTG
Sequence NM_001099930.1  ..................................................

                               460       470       480       490       500
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  GGCATGGCCGTCTTCCTGAAGAATGTGACCTTCCACGGGATCCTGCTGGA
Sequence NM_001099930.1  ..................................................

                               510       520       530       540       550
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  CTCGCTCTTTGAGGAGGGCGGCGCCACCTGGCAGGAGGTGTCGGAGCTGC
Sequence NM_001099930.1  ..................................................

                               560       570       580       590       600
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon D  TGAAGGCGGGCATCCAGGAGGGCGTGGTGCAGCCGCTCAAGTGCACCGTG
Sequence NM_001099930.1  ..................................................

                               610       620       630       640
                       ....|....|....|....|....|....|....|..
Final Contig Amplicon D  TTCCCCAGGACCAAGGTGGAGGCCGCCTTCCGCTACATGGCC
Sequence NM_001099930.1  ..................................
```

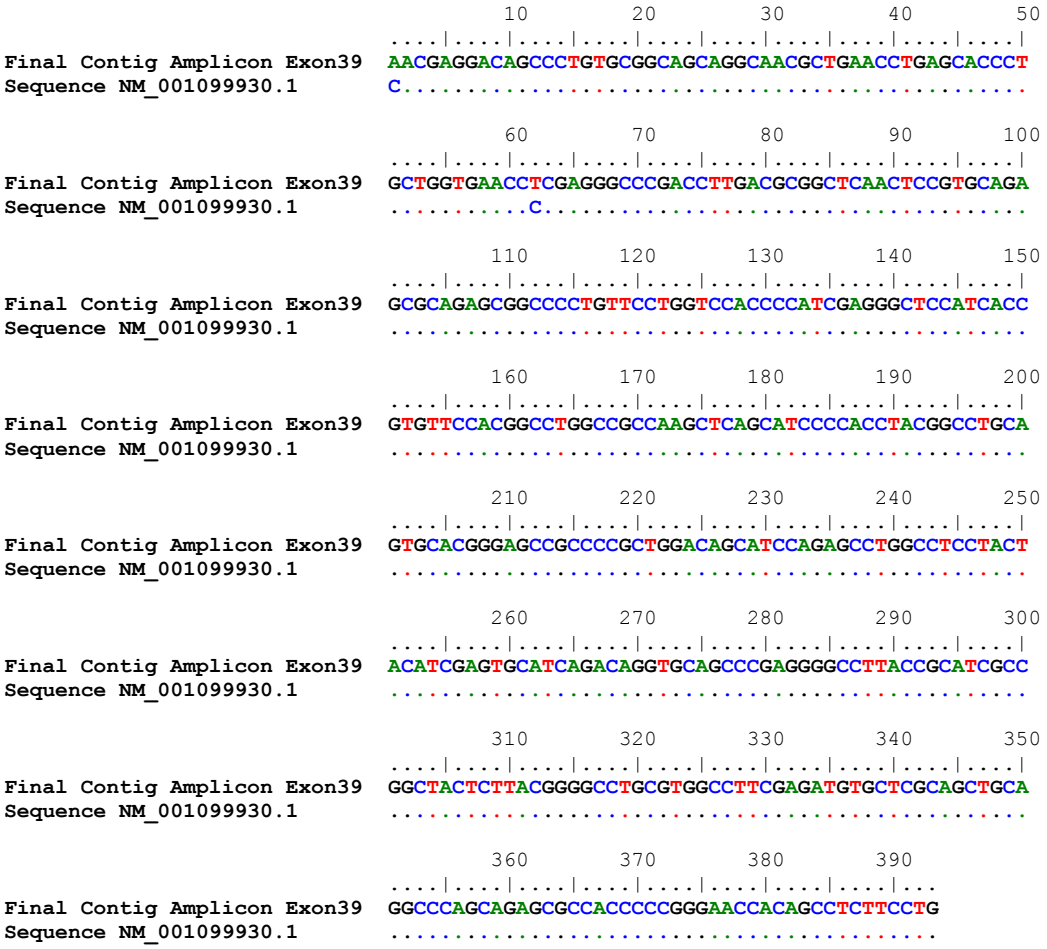
Amplicon L:


10 20 30 40 50
Final Contig Amplicon L GGAGGGCGGCGCCACCTGGCAGGAGGTGTCGGAGCTGCTGAAGGCGGGCA
Sequence NM_001099930.1
60 70 80 90 100
Final Contig Amplicon L TCCAGGAGGGCGTGGTGCAGCCGCTCAAGTGCACCGTGTTCCCCAGGACC
Sequence NM_001099930.1
110 120 130 140 150
Final Contig Amplicon L AAGGTGGAGGCCGCCTTCCGCTACATGGCCCAGGGCAAGCACATAGGCAA
Sequence NM_001099930.1
160 170 180 190 200
Final Contig Amplicon L AGTGGTCATCCAGGTGCGCGAGGAGGAGCAGGGGCCGGCGCCGCGCGGGC
Sequence NM_001099930.1
210 220 230 240 250
Final Contig Amplicon L TGCCGCCCATCGCATTGACCGGCTTGTCCAAGACCTTCTGCCCCCCCCAC
Sequence NM_001099930.1
260 270 280 290 300
Final Contig Amplicon L AAGAGCTACGTCATCACCGGGGGCCTGGGCGGCTTCGGCCTGCAGCTGGC
Sequence NM_001099930.1
310 320 330 340 350
Final Contig Amplicon L GCAGTGGCTCCGGCTGCGAGGGGCCCAGAAGCTGGTGCTCACCTCCCGCT
Sequence NM_001099930.1
360 370 380 390 400
Final Contig Amplicon L CGGGCATCCGCACAGGCTACCAGGCCAGGCAGGTCCGAGAGTGGAGACGC
Sequence NM_001099930.1
410 420 430 440 450
Final Contig Amplicon L CAGGGCGTCCAGGTCCTGGTGTCCACCAGCAACGCCAGCTCGCTGGACGG
Sequence NM_001099930.1
460 470 480 490 500
Final Contig Amplicon L CGCTCGGAGCCTCATCACTGAGGCCACACAGCTTGGGCCCGTGGGAGGCG
Sequence NM_001099930.1
510 520 530 540 550
Final Contig Amplicon L TCTTCAACCTGGCCATGGTCCTGAGAGACGCCGTGCTGGAGAACCAGACC
Sequence NM_001099930.1
560 570 580 590 600
Final Contig Amplicon L CCGGAGTTCTTCCAGGACGTCAGTAAGCCCAAGTACAGCGGCACCGCGAA
Sequence NM_001099930.1
610 620 630 640 650
Final Contig Amplicon L CCTGGACAGGGTGACCCGGGAGGCGTGTCCCGAGCTGGACTACTTCGTGA
Sequence NM_001099930.1
660 670 680 690 700
Final Contig Amplicon L TCTTCTCCTCCGTGAGCTGCGGGCGCGGCAATGCCGGCCAGGCCAACTAC
Sequence NM_001099930.1
710 720 730 740
Final Contig Amplicon L GGCTTCGCCAACTCTGCCATGGAGCGCATCTGCGAGAAGCGC
Sequence NM_001099930.1

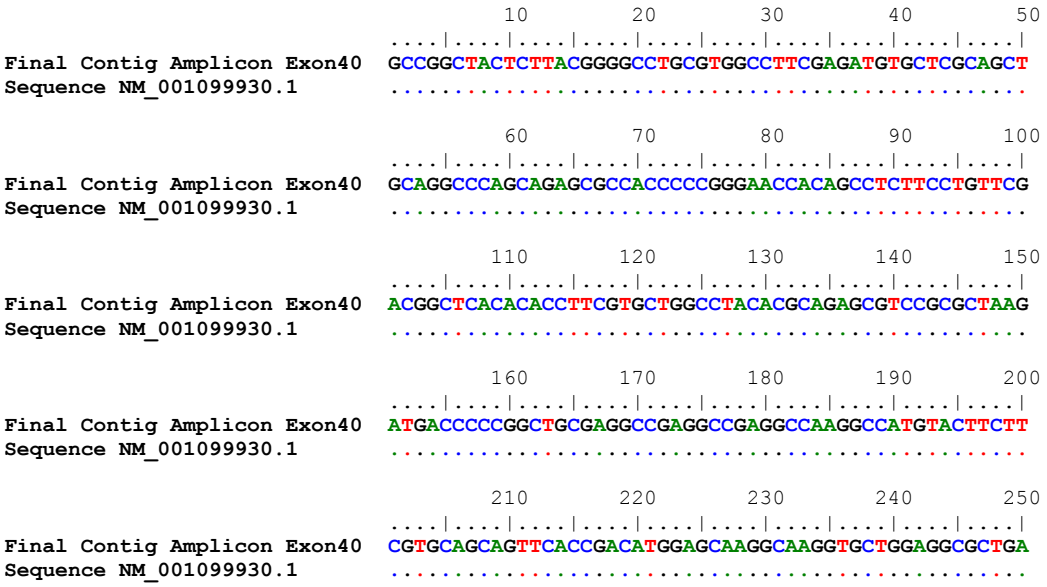
Amplicon Q:

```
                                10        20        30        40        50
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q GCCATGGAGCGCATCTGCGAGAAGCGCCGGCACGACGGGCTCCCAGGCCT
Sequence NM_001099930.1 ..................................................

                                60        70        80        90       100
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q CGCCGTGCAGTGGGGTGCGATCGGCGACGTGGGCGTCGTCCTGGAGACCA
Sequence NM_001099930.1 ..................................................

                               110       120       130       140       150
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q TGGGCACCAACGACACGGTCATCGGCGGGACGCTGCCCCAGCGCATCGCC
Sequence NM_001099930.1 ..................................................

                               160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q TCCTGCCTGGAGGTGCTGGATCTCTTCCTGAGCCAGCCCCACCCCGTCCT
Sequence NM_001099930.1 ..................................................

                               210       220       230       240       250
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q GAGCAGCTTCGTCCTGGCTGAGAAGAAGGCTGCCGCCCCCAGGGACGGCA
Sequence NM_001099930.1 ....................C.....................

                               260       270       280       290       300
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q GCAGCCAGAAGGACCTGGTCAAGGCCGTGGCTCACATCCTGGGCATCCGG
Sequence NM_001099930.1 ..................................................

                               310       320       330       340       350
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q GACGTGGCCTCCATCAATCCGGACAGCACGCTGGTGGACCTGGGCCTGGA
Sequence NM_001099930.1 ..................................................

                               360       370       380       390       400
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q CTCGCTCATGGGCGTGGAGGTGCGCCAGATACTGGAGCGAGAGCACGACC
Sequence NM_001099930.1 ..................................................

                               410       420       430       440       450
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q TGGTGCTGTCCATGCGGGAGGTGCGGCAGCTCAGCCTCCGGAAGCTACAG
Sequence NM_001099930.1 ..................................................

                               460       470       480       490       500
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q GAACTCTCCTCGAAGACCAGCACGGACGCCGACCCGGCGACTCCCACATC
Sequence NM_001099930.1 ..................................................

                               510       520       530       540       550
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q CAACGAGGACAGCCCTGTGCGGCAGCAGGCAACGCTGAACCTGAGCACCC
Sequence NM_001099930.1 .C................................................

                               560       570       580       590       600
                       ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Q TGCTGGTGAACCCCGAGGGCCCGACCTTGACGCGGCTCAACTCCGTGCAG
Sequence NM_001099930.1 ..................................................

                               610
                       ....|....|....|
Final Contig Amplicon Q AGCGCAGAGCGGCCC
Sequence NM_001099930.1 ...............
```

Amplicon exon39:



Amplicon exon40:



```
                                  260       270       280       290       300
                         ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon Exon40  TACCGCTCCAGGGCCTGGAGGCGCGCGTGGCGGCCACCGTGGACCTGATC
Sequence NM_001099930.1       ..........................................................

                                  310       320       330
                         ....|....|....|....|....|....|....|....
Final Contig Amplicon Exon40  ACGCAGAGCCACGCGGGCCTGGACCGCCACGCCCTCAGC
Sequence NM_001099930.1       ........................................
```

Amplicon F:

```
                                   10        20        30        40        50
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   ACACACCTTCGTGCTGGCCTACACGCAGAGCGTCCGCGCTAAGATGACCC
Sequence NM_001099930.1   ..................................................

                                   60        70        80        90       100
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   CCGGCTGCGAGGCCGAGGCCGAGGCCAAGGCCATGTACTTCTTCGTGCAG
Sequence NM_001099930.1   ..................................................

                                  110       120       130       140       150
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   CAGTTCACCGACATGGAGCAAGGCAAGGTGCTGGAGGCGCTGATACCGCT
Sequence NM_001099930.1   ..................................................

                                  160       170       180       190       200
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   CCAGGGCCTGGAGGCGCGCGTGGCGGCCACCGTGGACCTGATCACGCAGA
Sequence NM_001099930.1   ..................................................

                                  210       220       230       240       250
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   GCCACGCGGGCCTGGACCGCCACGCCCTCAGCTTCGCCGCTCGCTCCTTC
Sequence NM_001099930.1   ..................................................

                                  260       270       280       290       300
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   TACCAGAAGCTGCGCGCCGCCGAGAACTACTGGCCGCAGGCCACCTACCA
Sequence NM_001099930.1   ..................................................

                                  310       320       330       340       350
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   CGGCAACGTGACGCTGCTGCGCGCCAAGACGGGCGGCGCCTACGGCGAGG
Sequence NM_001099930.1   ..................................................

                                  360       370       380       390       400
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   ACCTGGGCGCCGACTACAACCTGTCGCAGGTGTGCGACGGCAAGGTCTCG
Sequence NM_001099930.1   ..................................................

                                  410       420       430       440       450
                          ....|....|....|....|....|....|....|....|....|....|
Final Contig Amplicon F   GTGCACGTCATCGAGGGCGACCACCGCACGCTGCTGGAGGGCAGCGGCCT
Sequence NM_001099930.1   ..................................................


                          ....
Final Contig Amplicon F   GGAG
Sequence NM_001099930.1   ....
```