Deliverable Report

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Objective

The objective of this study was to examine the role of vitamin D on the development of Systemic Granulomatosis (SG).

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Introduction

Meagre (*Argyrosomus regius*) is an emerging species for the diversification of the European aquaculture, due to its attractive and competitive biological attributes. These include a fast growth rate of ~1 kg per year, low Feed Conversion Ratio of 0.9-1.2, low fat content and excellent taste (Monfort, 2010; Duncan *et al.,* 2013).

One of the most important bottlenecks of meagre production is Systemic Granulomatosis (SG), a pathological condition affecting the majority of farmed populations. Systemic granulomatosis is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. The aetiology of the disease is unknown, however it is suspected that it is related to nutritional factors (Katharios *et al.,* 2011). One of the objectives of Work Package 24 (WP24) is to identify potential nutritional causes of SG via several feeding trials. The first of these trials comprises the investigation of the effect of increasing levels of vitamin D fed at 4 different levels in the development of SG.

Vitamin D_3 is a fat-soluble pro-hormone, best known for its role in calcium and phosphate homeostasis and in protecting skeletal integrity (DeLuca, 2004). However, in the past few decades new research studies demonstrated that the vitamin D_3 endocrine system plays an important role in other biological processes not related to calcium and phosphorus homeostasis, such as muscle function, autoimmune diseases, cardiovascular physiology, cell proliferation and differentiation (Holick, 2003; Sutton & MacDonald, 2003). In terrestrial vertebrates, it is synthesized in the skin under the influence of ultraviolet light from the sun or it is obtained from food. In contrast, fish do not synthesize vitamin D_3 and are fully dependent on dietary sources (Lock *et al*., 2010).

Vitamin D_3 requires transformations to become bioactive. These transformations involve cytochrome P450 (CYP) enzymes that hydroxylate the steroid (vitamin D) to several metabolites. As in mammals, two hydroxylations of D_3 take place in fish (Lock *et al*., 2010). First, vitamin D_3 is hydroxylated in the liver to produce the intermediate 25-hydroxycholecalciferol or 25-hydroxyvitamin D_3 [$(25(OH)D_3$]. The proper enzyme activity responsible for this 25-hydroxylation is still rather enigmatic even in mammals; several candidate enzymes with 25-hydroxylase ability and broad substrate specificity have been proposed (Aiba *et al*. 2006). One example is the sterol 27-hydroxylase (CYP27A1), which carries out the 25-hydroxylation of vitamin D_3, as well as the 27-hydroxylation of cholesterol in bile acid biosynthesis (Ibarra *et al*. 2004). Similar functional CYP27A1 has been isolated and characterized in gilthead sea bream (*Sparus aurata*) that governs D_3 metabolism (Bevelander *et al*., 2008). The second hydroxylation takes plays in liver, kidneys and other tissues to produce the most active metabolites viz. 1,25-hydroxyvitamin D_3 (calcitriol) ($1,25(OH)_2D_3$) and 24,25-dihydroxycholecalciferol ($24,25(OH)_2D_3$) (Lock *et al*., 2010). Research in mammalian kidney has revealed that CYP27B1 makes calcitriol and CYP24 produces 24,25-dihydroxycholecalciferol. However, similar information in fish is lacking. Moreover, elevation of $25(OH)D_3$ plasma levels in mammals has been associated with hypervitaminosis D. However, plasma levels in fish can be misleading as fish store large quantities of vitamin D_3 in their liver and fatty tissues without metabolizing it and still hydroxylation takes place in the liver without the appearance of a precursor in the plasma ((Lock *et al*., 2010). In addition, local production of the most polar metabolites has been demonstrated in a variety of tissues (Graff *et al*., 1999). Thus, in fish, the physiological activity of $25(OH)D_3$ has been questioned since its discovery (Nahm *et al*., 1979).

In mammals, 25(OH)D_3 is likely to act as a membrane antioxidant by stabilizing the membrane against lipid peroxidation *via* an interaction between its hydrophobic ring; the other antioxidant activity of vitamin D_3 seems to be its effect on antioxidant enzymes (Wiseman, 1993). However, no studies have been conducted on antioxidant effects of vitamin D_3 in fish. Most research on vitamin D_3 has been carried out in mammals and birds, whereas studies in fish are scarce. Most of these refer to the effect of vitamin D_3 on growth (Andrews *et al.,* 1980, Graff *et al*., 2002), calcium and phosphorus metabolism (Fenwick *et al.,* 1984, Hilton & Ferguson, 1982), ontogenesis and ossification (Darias *et al.,* 2010, 2011), and immune parameters (Cerezuela *et al*., 2009). The origin and hydroxylation of vitamin D metabolites, as well as the significance for the fish is reviewed by Lock *et al*. (2010).

The purpose of this trial was to examine whether vitamin D_3 affects the development of SG, but also to investigate its role in blood serum biochemistry, antioxidant system and calcium/phosphorus metabolism in meagre.

Materials and methods

Experimental diets

Four experimental diets with increasing levels of vitamin D_3 were prepared at the HCMR laboratory in Athens, Greece. The basal diet was formulated to contain about 53% crude protein and 13% crude lipid. Vitamin D_3 was supplemented separately to the basal diet of the mixture to obtain concentrations of 4550 (D0), 7000 (D1), 10000 (D2) and 20000 (D3) IU kg^{-1} respectively (**Table 1**).

Table 1. Formulation of the experimental diets (% dry weight)

Ingredient	***D0***	***D1***	***D2***	***D3***
Soyabean meal	10	10	10	10
Fish meal	50	50	50	50
Wheat	17.4	16.9	16.3	14.3
Corn gluten	14.6	14.6	14.6	14.6
Fish oil	7.5	7.5	7.5	7.5
Premix	0.5	0.5	0.5	0.5
Vitamin D (suppl)		0.49	1.1	3.1
Vitamin D (IUKg^{-1})	4550	7000	10000	20000

Experimental fish and feeding trial

Three-month old meagre, produced in May 2014 at the facilities of HCMR, Crete, Greece were used for the feeding trial. In total, 600 fish were weighed and placed into 12 x 500-l cylindrical tanks at an initial density of 50 fish per tank (0.47 kg m^{-3}). Three replicates were used for each diet. Prior to the commencement of the experiment, the fish had been acclimated for 1 week by being fed on the D0 diet. The feeding trial lasted 93 days (July 2014-October 2014).

Tanks were supplied with borehole water (salinity 35‰, pH 7.5) and all had similar light conditions and temperature (20°C). Air-stones in each tank provided aeration and nets were placed over the tanks in order to prevent fish escape. The fish were hand-fed to visual satiety twice a day for 7 days a week, while feed intake was recoded daily.

Sampling and analytical methods

Before every sampling, the fish were starved for 24 h to reduce handling stress and allow digestion and tract evacuation. Fish were then anaesthetized with 2-phenoxyethanol and weighed. **Table 2** shows the number of fish used for every analysis at the four samplings that were performed during the 3-month feeding trial. At the end of the feeding trial, the following performance indicators were calculated:

Condition factor (K)	$K = \frac{BW\ (g)}{TL\ (cm)^3}$
Specific Growth Rate (SGR)	$SGR\% = 100x\ \frac{\ln(Final\ BW - Initial\ BW)}{\Delta T}$
Feed conversion ratio (FCR)	$FCR = \frac{feed\ intake\ (g)}{wet\ weight\ gained\ (g)}$

BW: body weight, TL: Total Length, ΔT: (Time, days)

Table 2. Number of fish / diet sampled for every analytical method performed

Sampling	*Evaluation of granulomas*	*Histology*	*Plasma analysis*	*Vitamin D determination*	*Specific biomarkers*
Initial	8	8	-	50	50
1st month	10	10	-	-	-
2nd month	10	10	-	36	36
3rd month	40	10	15	36	36

Visual evaluation of Systemic Granulomatosis

To assess fish status regarding the presence of granulomas, a semi-quantitative method was developed based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. Each fish was dissected and internal organs were examined macroscopically. Fresh squash preparations of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney were assessed under a stereoscope. For the general state of each individual, the sum of the scores from the various tissues was calculated. The assessment scale used was according to the following scoring system:

Score 0	No granulomas
Score 1	Granulomas visible only with microscopy
Score 2	Granulomas visible macroscopically
Score 5	Tissue calcification

Histology

Samples of heart, liver, intestine and kidney were fixed in 4% formaldehyde: 1% glutaraldehyde (McDowell & Trump 1976) for at least 24 h. Subsequently, they were dehydrated in gradually increased ethanol solutions (70-96%) and then embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections of 4 μm were obtained with a microtome (RM 2035, Leica, Germany). After drying, slides were stained with methylene blue/azure II/basic fuchsin according to Bennett *et al.* (1976) and examined under a light microscope. Further to routine stains, several sections were stained with PAS, Alizarin and Ziehl-Neelsen stains.

Plasma analysis

Blood samples were collected from the caudal vasculature into heparinized syringes. After centrifugation for 15 min at 6000 r.p.m., the plasma was removed and stored at −20°C for further analysis. Specifically, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), phosphorus (P), magnesium (Mg), triglyceride (Trig), total cholesterol (Chol), lactate dehydrogenase (LDH), glucose (Glu) and total proteins (TP) were assessed using commercially available kits (Biosis, Greece). The samples were pooled by diet and by the severity of granulomas (no granuloma, granuloma and calcification). Analyses were performed using a BA-88A Semi-Auto Chemistry Analyzer.

Determination of supplemented vitamin D_3 in diets and whole fish body

Total vitamin D_3 in triplicate samples of all diets (D0-D1-D2-D3) and whole bodies of nine fish per dietary group (D0-D1-D2-D3) sampled at the end of the experiment were determined with LC/MS/MS according to Lipkie *et al*. (2013).

Calcium (Ca) and Phosphate (PO_4^{3-}) assays in liver tissue

Nine fish per dietary group (D0-D1-D2-D3) sampled at the end of the experiment were frozen in dry ice and stored at -80°C until dissection of liver. 60 mg of liver tissues were homogenized in 1 ml Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl) pH 7.4 and centrifuged at 10.000 g (4°C) for 15 min. Measurements of Ca and PO_4^{3-} were performed according to commercial kits (Cayman Chemicals). The Ca assay utilizes an optimized variant of the well-established o-Cresolphthanlein-Ca reaction which in alkaline environment forms a complex with a purple color. The intensity of the color is directly proportional to the concentration of Ca in the sample. The Phosphate assay is based on the complex formed between malachite green molybdate and free orthophosphate under acidic conditions. It detects and quantifies inorganic free phosphate; lipid-bound or protein bound phosphates must first be hydrolyzed and neutralized prior to measurements.

Antioxidant enzymes (SOD, CAT, GSH) in liver tissue

Nine fish per dietary group (D0-D1-D2-D3) sampled at the end of the experiment were frozen in dry ice and stored at -80°C until dissection of liver. For SOD and CAT activities liver tissues of 500 mg were homogenized in 0.1 M potassium phosphate buffer (0.125 M KCl, 1m

M DDT, 1 mM EDTA, 0.1 mM PMSF) pH 7.4 and were centrifuged at 10.000 g (4°C) for 20 min. SOD activity was measured in the supernatant by using xanthine/xanthine oxidase model as the source of superoxide radicals with a water-soluble tetrazolium salt (4-[3—(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]1-3benzene disulfanate) according to Peskin and Winterbourn (2000) using a commercial kit (Cayman Chemicals). CAT activity was estimated by measuring residual hydrogen peroxide after incubation with the enzyme and addition of ferrous ions and thiocyanate according to Cohen *et al.* (1996). For GSH content 50 mg liver from 9 fish per dietary group (D0-D1-D2-D3) sampled at the end of the experiment were homogenized in 650 µl of ice-cold 5% metaphosphoric acid and 0,6 % sulfosalicylic acid and centrifuged at 3000 g for 10 min at 4 °C. Supernatants were collected and assayed according to Rahman *et al*. (2006). The assay is based on the reaction of GSH with DTNB (Ellman's reagent) that produces a chromophore (TNB), which oxidizes GSH-TNB adduct (GS-TNB). The rate of formation of TNB is proportional to the GSH concentration in the sample. The product GS-TNB is then reduced by glutathione reductase in the presence of NADPH. The amount of GSH measured represents the sum of reduced and oxidized GSH in the sample.

Vitamin D_3 metabolizing enzymes (CYP27, CYP24)

Sequencing of the Cyp27 and CYP24

The sequence of Cyp27 mRNA for meagre (*Argyrosomus regius)* was unavailable and was partially determined using a Smarter RACE cDNA amplification Kit (Takara) according to manufacturer's recommendations and primers designed based on conserved regions of Cyp27 mRNA coding region in other fish species:
CYPF_1100-1120 GTGGCAGCCTCATCAGTCC,
CYPF_1100-1120_SC AATGCTATGTGCGGCTGACC,
CYPF_1840-1860 GAGACCCTTGTGTAGACTCCA,
CYPR_1500-1520 TGGGTCAGCCGCACATAGC,
CYPR_2000-2020 GGACCTCTGCAGGACCACG.
The sequence of Cyp24 mRNA was unavailable for meagre as well as for other fish species; thus it was not possible to be determined.

RNA extraction and cDNA synthesis

Nine fish per dietary group (D0-D1-D2-D3) were frozen in dry ice and stored at -80°C until dissection of liver and extraction of RNA. RNA was extracted from approximately 20 µg of tissue per sample using the TRI© Reagent (Sigma) and according to manufacturer's instructions. To eliminate gDNA contamination, RNA preparations were then treated with DNAse I (New England Biolabs) at 37°C for 30 min, followed by heat inactivation of the enzyme and ethanol-precipitation with ammonium acetate, according to manufacturer's recommendations. RNA concentration and purity was assessed by Quawell-Q5000 micro volume cuvette-free spectrophotometer. cDNA was synthesized from 500 ng total RNA per sample using the PrimeScript RT reagent kit (TAKARA) following the protocol recommended by the manufacturer for SYBR green assay.

Quantitative RT-PCR analysis

The relative transcript abundance of CYP27 was determined by quantitative (q) RT-PCR. 10 µl reactions with the equivalent of ca. 15 ng total RNA were performed in duplicates for each sample using the KAPA SYBR fast qPCR Universal Master Mix (KapaBiosystems),

following the manufacturer's instructions, on an ABI 7500 real time PCR system (Applied Biosystems/Life Technologies). Expression levels of CYP27 for each sample were normalized by the corresponding expression of β-Actin. The primers used for the qRT-PCR were: for β-Actin CGCGACCTCACAGACTACCT and AACCTCTCATTGCCGATG (M. Pavlidis and A. Tsalafouta, personal communication) and for Cyp27 ACCCGTACAGCTTCATCC and TCGTATTGCTGCATTAACCTG. Primers for Cyp27 were designed based on partial cDNA sequence determined as described above using the PerlPrimer software. β-Actin and Cyp27 primers were used at 200 nM and 400 nM final concentration respectively. Cycling conditions for both genes were as follows: a 3 min initial denaturation step at 95°C followed by 40 cycles of denaturation for 3 sec at 95°C and primer annealing and template extension for 30 sec at 60°C. PCR amplification efficiencies and Ct-quantity regression coefficient (R^2) for β-Actin and Cyp27 were 95%/0.997 and 98%/0.996 respectively. Relative transcript abundance for each gene and cDNA sample was determined based on Ct values with the 7500 software v2.3 (Applied Biosystems, USA). Transcript relative quantity for CYP27 was normalized by corresponding quantity of β-actin in each sample. Means and standard errors of normalized Cyp27 relative expression were determined per treatment.

Statistical analysis

Data on blood parameters, vitamin D_3 in diets and fish body as well as calcium, phosphates, antioxidant enzymes activities and expression of CYP27A in the liver were analyzed by One-Way Analysis of Variance (ANOVA) to test any differences among the four experimental diets. The statistical significant level was set at $p<0.05$. Tukey–Kramer post hoc test was used to rank the groups for blood parameters, while Tukey post hoc test was for all the others. Kruskal-Wallis test was performed to compare the severity of granulomatosis in the various organs among groups as well as the overall condition of the fish. Two tailed Pearson correlation was used for comparative analyses between: (1) percentage of granulomas to relative expression of CYP27, (2) percentage of granulomas to calcium concentration and (3) relative expression of CYP27 to calcium concentration. Statistical analyses were made using SPSS Statistics 20 software.

Results

Vitamin D_3 determination

Vitamin D_3 content of fish (**Table 3**) was correlated with the dietary vitamin D_3 levels (**Figure 1**). A significant difference was observed for fish fed on D2 and D3 ($P<0.05$).

Table 3. Estimates of vitamin D_3 in diets and whole fish body in mg/kg (IU/ kg).

Diet	***Dietary Content***	***Fish Body content***
D0	0.114 (4550)	0,109 (4380)
D1	0,175 (7000)	0,117 (4668)
D2	0,250 (10000)	0,132 (5288)
D3	0,500 (20000)	0,188 (7532)

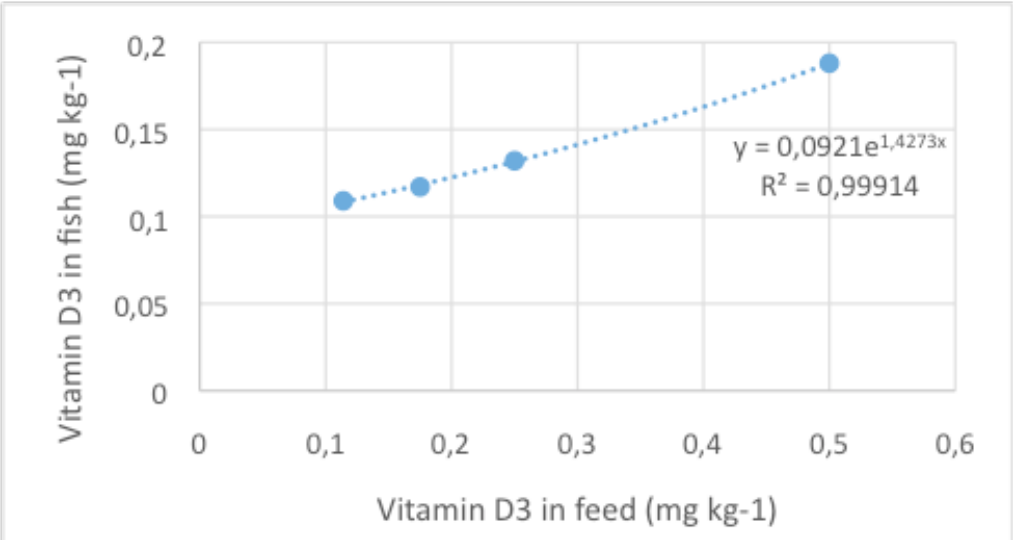

Figure 1. Correlation of vitamin D_3 in the diets and the vitamin D_3 measured in fish (whole body). There is a significant correlation (R^2: 0.999)

Growth performance

Juvenile meagre grew from ~4 g to ~35 g over the course of the feeding trial. The weights did not vary significantly among fish groups in any time point during the 12 weeks of the experiment (**Figure 2**). Significant differences between the final lengths of the fish among the diet groups were not observed. However the condition factor (K) of fish fed the D3 diet was significantly higher. Specific growth rate (SGR) and feed conversion ratio (FCR) didn't show any significant differences among the diets, although the tendency was an increase in FCR with the increase of dietary vitamin D_3 content (**Figure 3**).

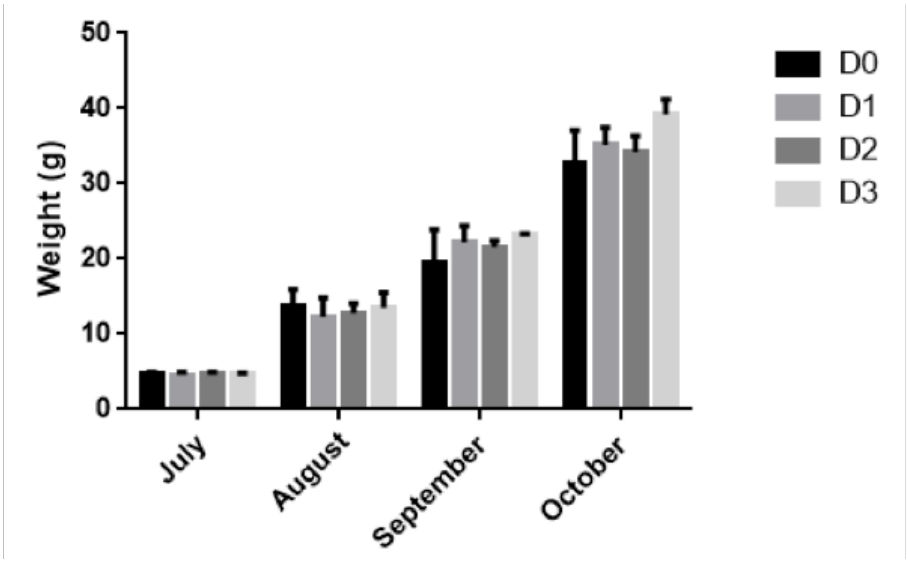

Figure 2. Average weights (g) of meagre fed the diets with different levels of vitamin D_3 in the beginning of the experiment and after 1, 2 and 3 months. Values are mean ± SD. No statistically significant differences were detected between the weights of the 4 dietary groups in any of the sampling points ($p>0,05$).

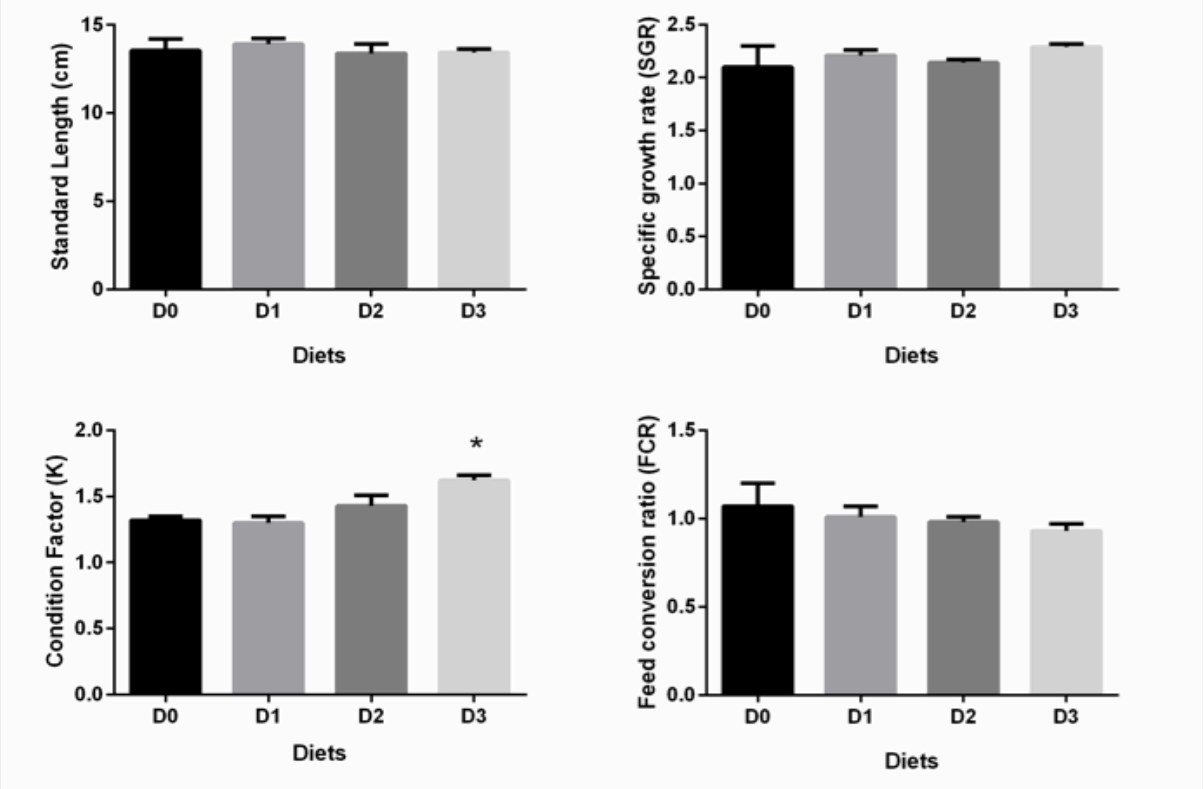

Figure 3. Average standard length (cm), Specific Growth Rate, Condition Factor and Feed Conversion Rate of meagre fed the diets with different levels of vitamin D_3 at the end of the feeding trial. Values are means ± SD. Asterisk indicates statistically significant differences between the diets ($p < 0,05$).

Evaluation of granulomas

At the beginning of the feeding trial the majority of the fish did not exhibit granulomas in the tissues studied. After 3 months of feeding, granulomas were observed in all groups of fish. The overall condition of the fish assessed as the sum of the tissues' score was not affected by vitamin D_3 supplementation. **Figure 4** shows the boxplots of total scores of granulomas for the four different diets at the end of the experiment. The medians of the four groups are similar which was confirmed by Kruskal–Wallis test ($H(3) = 3.55$, $p = 0.315$).

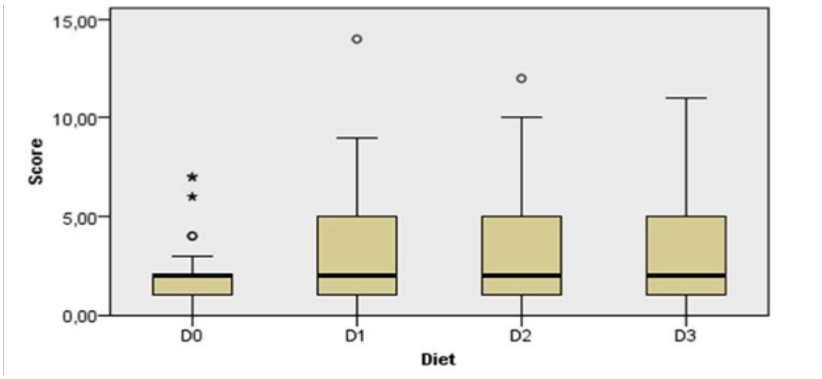

Figure 4. Boxplots of meagre's total score of granulomas at the end of the experiment for the four different diets. Outliers presented as circles and extreme scores as asterisks.

The tissues that appear to be affected first from SG are the kidney and the liver (first month of the feeding trial). The other soft tissues exhibit granulomas after the second month of the feeding trial when the fish are about 20 g. **Figure 5** and **6** show the percentage of fish in each of the 4 categories of the granulomas scoring system, at the end of the experiment for every examined tissue. A statistically significant difference exists between the diets and the development of SG only for the liver (p=0.023). The best diet was found to be the D0 while the worst for the appearance of granulomas was the D2 for the liver. The development of SG was not affected by vitamin D_3 for the other soft tissues (p>0,05). Kidneys and livers from the fish of all diets presented all granuloma categories: no granuloma, granulomas visible only with microscopy, granulomas visible macroscopically and tissue calcification.

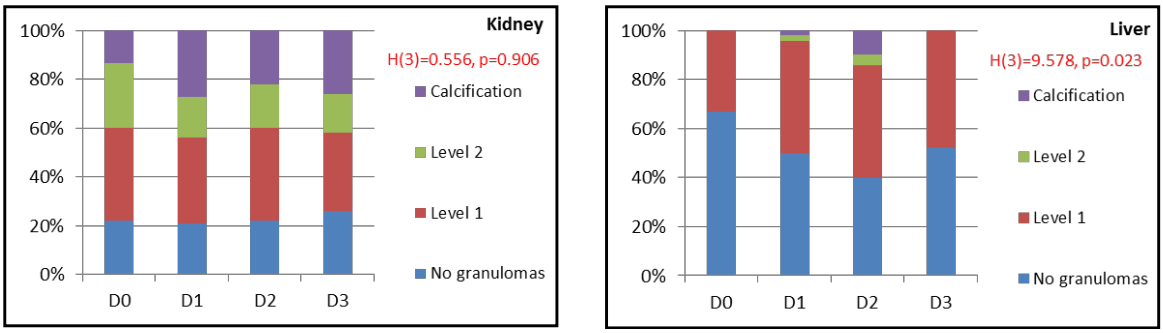

Figure 5. Granulomatosis severity in the kidneys and livers of the fish fed with various vitamin D_3 levels. Significant differences were indicated only in the liver. Kruskal-Wallis test results are indicated with red letters.

Tissue calcification was not observed in the heart, intestine, spleen, swim bladder and peritoneum in any condition tested. More than 80% of the fish of all diets had no granulomas on those tissues and only few of them had granulomas mainly visible by microscopy.

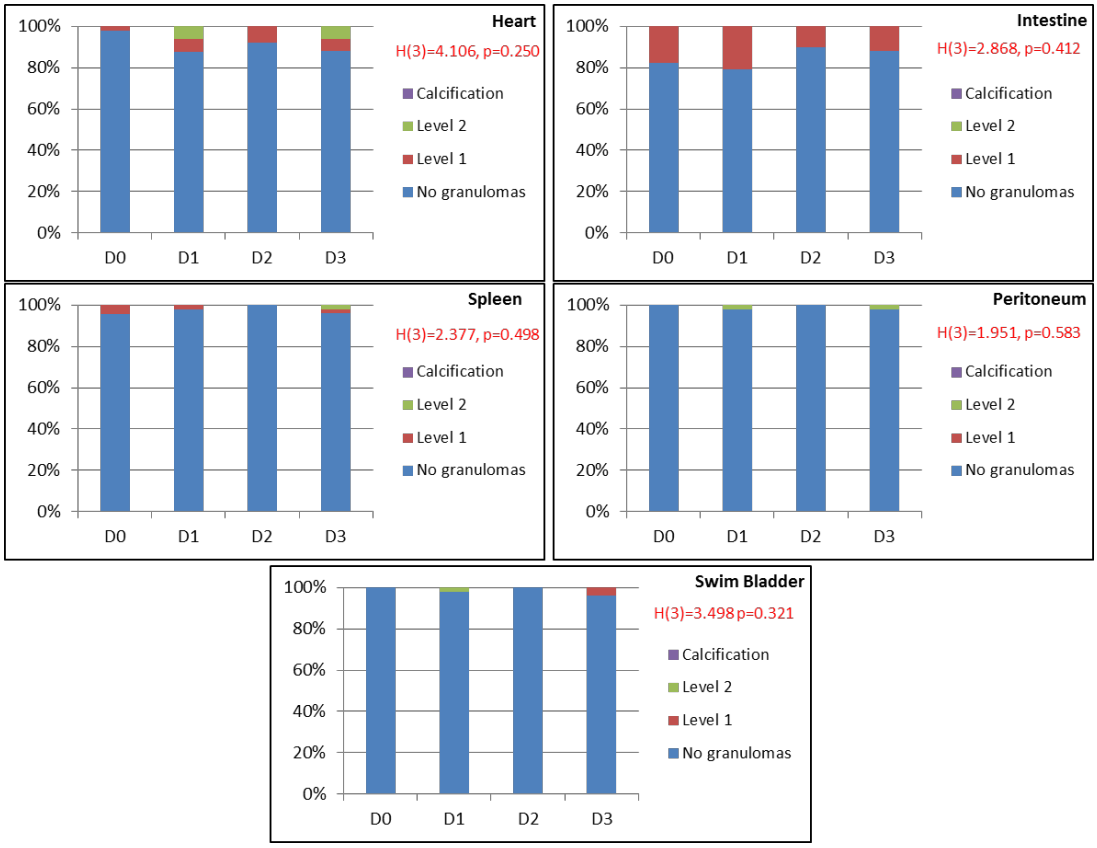

Figure 6. Granulomatosis severity in the organs of meagre fed with various vitamin D_3 levels. No significant differences were found between diets in these organs. Kruskal-Wallis test results are indicated with red letters.

Histology

Granulomas were defined by the presence of epithelioid cells which are macrophages resembling epithelial cells. The overall pathology was not different to that described by Katharios *et al*. (2011). Several stages of the granuloma formation can be identified in the examined tissues ranging from immature granulomas (**Figure 7**), multilayer mature granulomas to big nodules, possibly a result of the merging of several adjacent granulomas with big areas of central necrosis with dystrophic calcification circumscribed by fiber tissue. In several cases, mainly in livers, the initial stages of the granulomas are located at the blood vesicles resembling vasculitis (**Figure 8**). In these cases there is also an involvement of rodlet cells (**Figure 9**). Rodlet cells are present in large numbers in all tissues. Rodlets are aligned like epithelial cells in the peritoneal membranes but they are also found in livers, pancreas and intestine. Kidneys and livers are the organs mainly affected although granulomas are also found in hearts. Most of the livers are characterized by lipoid degeneration (**Figure 8A**) and in several cases there where proteinaceous-like deposits in the hepatic parenchyma (**Figure 8B**). In most of the cases the granulomas do not contain necrotic areas, however there are cases where necrosis is evident and in one case there was extensive caseous necrosis in the kidney well circumscribed by fibrous tissue (**Figure 7B**). The fish of all different experimental groups had similar lesions and the histological assessment was in accordance to the visual examination of the fresh preparations.

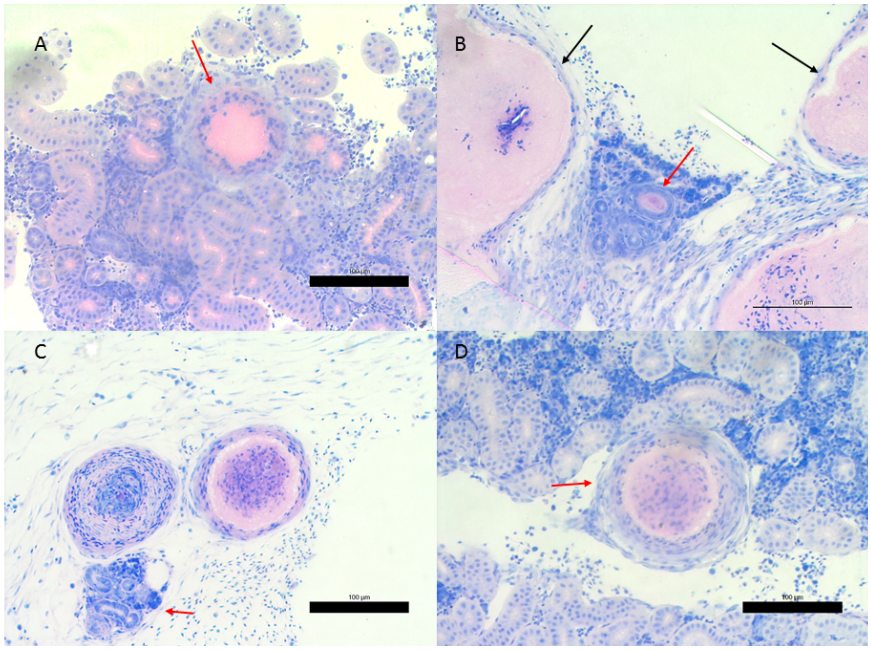

Figure 7. A. Immature granuloma in the kidney of a fish of D2 group. There is an amorphous, acellular area, which is surrounded by inflammatory cells. **B.** An immature granuloma in the kidney (red arrow) in a small area of a kidney with normal appearance. In this particular fish there was extensive caseous necrosis in this organ, a small part of which is indicated with black arrows. **C.** Two adjacent granulomas sectioned at different levels over a small part of renal tissue (red arrow). **D.** Typical appearance of a "young" granuloma in kidney.

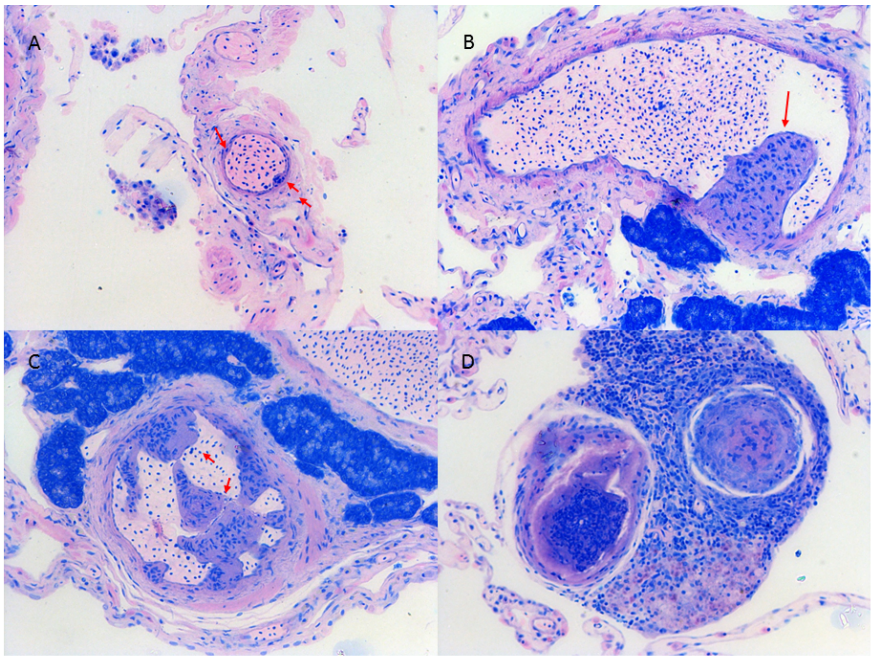

Figure 8. Blood vessel implication is evident in the manifestation of the disease. Various sections of blood vessels from the peritoneal membranes and the liver of affected fish are shown. There are specific growths composed of inflammatory cells at the endothelium of the vessels, which are indicated with red arrows. In more progressed stages (C and D) these growths seem to block the lumen of the vessel.

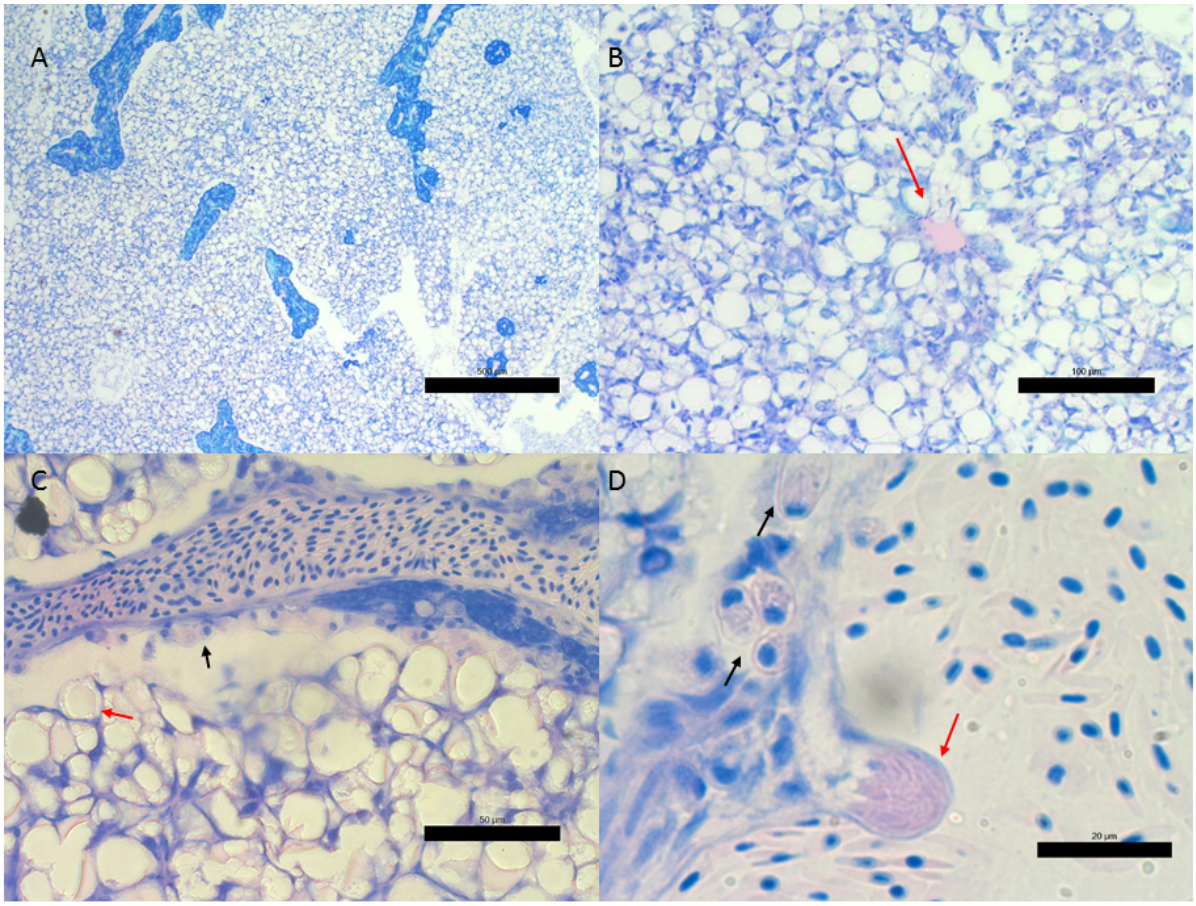

Figure 9. A. Lipoid liver degeneration is evident in almost all fish examined. **B.** The red arrow points to an amorphous proteinaceous deposit in liver parenchyma. This can be related to the development of granulomatosis. **C.** In advanced cases of lipoid liver degeneration there are hyaline accumulations within the vacuoles of the hepatocytes (red arrow). Rodlet cells were infiltrated in the outer part of the blood vessels (black arrow). **D.** Higher magnification of a blood vessel of the liver showing the infiltration of the rodlet cells (black arrows). The textural appearance of the growth indicated with the red arrow resembles the eosinophilic rodlets contained in the rodlet cells that can be discharged under specific conditions.

Plasma analysis

The only parameter that showed significant differences among fish fed the four vitamin D_3 levels was plasma glucose. Fish fed the D0 diet had the highest plasma glucose concentration, while the fish of D1 and D2 group had the lowest. The other serum parameters did not show significant differences among the groups of diets. **Table 4** shows the mean values ± SD of each plasma parameter per diet.

Table 4. Mean concentrations (±SD) of blood parameters in meagre fed four diets with 4550 (D0), 7000 (D1), 10000 (D2) and 20000 (D3) IU/Kg vitamin D_3, at the end of the experiment. Different letters (a, b) indicate statistically significant differences.

	Diets			
	D0	**D1**	**D2**	**D3**
Enzymes				
ALP (U/L)	139.5±16.7	139.0±24.5	124±12.6	135±15.9
AST (U/L)	96.7±9.6	81.8±12.9	81.2±8.8	100.2±21.9
ALT (U/L)	1.7±2.4	2.3±3.0	2.5±1.6	3.5±1.9
De Ritis ratio (AST/ALT)	38.8±7.2	26.5±14.6	45.1±27.3	37.1±21
Ions				
Ca (mmol/l)	1.6±0.6	1.7±0.6	1.5±0.2	1.4±0.2
P (mmol/l)	8.0±0.7	8.2±0.5	8.2±1.1	9.3±0.5
Mg (mmol/l)	0.8±0.1	0.8±0.1	0.8±0.1	0.7±0.1
Lipids				
Trig (mg/dl)	301.3±14.5	283.0±39.8	307.0±25.7	270.3±38.0
LDH (U/L)	72.1±19.8	62.7±8.1	59.4±4.8	55.4±9.7
CHOL (mmol/l)	3.3±0.7	3.6±0.7	3.0±0.8	3.3±0.9
Other				
Glu (mmol/l)	5.1±0.4[a]	3.2±0.9[b]	3.0±0.8[b]	3.6±0.7[a,b]
TP (g/l)	57.5±6.9	59.5±10.9	59.3±11.1	50.0±8.6
BUN (U/L)	10±0.8	11.0±1.2	11.5±2.5	10.9±1.0

Table 5 shows the mean values ± SD of the same plasma parameters grouped by the severity of the granulomas. The label no granuloma refers to score 0, granuloma to score 1 and 2 and calcification to score 5 of the scoring system used. Significant differences were observed on the enzymatic activity of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The ALT increases as the disease progresses to calcification, while ALP showed lower values in fish without granulomas compared with those with granulomas, but not in relation to those with calcification. AST showed higher value in fish with calcified tissues compared with those with no granulomas. The same pattern with AST was found also for BUN and LDH concentration while the total protein levels decreased in fish with tissue calcification compared with those with no granulomas.

Table 5. Mean concentrations (±SD) of blood parameters in meagre with no granulomas, granulomas and calcification of even one tissue at the end of the experiment. Different letters (a, b) show statistically significant differences between the three conditions.

	No granuloma	***Granuloma***	***Calcification***
Enzymes			
ALP (U/L)	120.3±12.1^{a}	147.5±16.5^{b}	136.0±14.5^{a,b}
AST (U/L)	77.3±9.9^{a}	93.4±9.0^{a,b}	99.3±19.0^{b}
ALT (U/L)	0.1±1.5^{a}	2.6±0.7^{b}	4.8±1.3^{c}
De Ritis ratio (AST/ALT)	67.1±8.3	37.8±8.1	23±7.8
Ions			
Ca (mmol/l)	1.4±0.4	1.5±0.4	1.8±0.4
P (mmol/l)	9.0±0.7	8.8±1.6	8.0±0.9
Mg (mmol/l)	0.7±0.1	0.8±0.1	0.8±0.1
Lipids			
Trig (mg/dl)	274.1±34.4	303.3±23.0	293.9±33.9
LDH (U/L)	53.3±6.8^{a}	63.0±13.1^{a,b}	70.8±12.3^{b}
CHOL (mmol/l)	3.0±0.8	3.5±0.5	3.4±0.7
Other			
Glu (mmol/l)	4.2±0.6	3.2±1.0	3.7±1.2
TP (g/l)	58.4±11.2^{a}	61.4±6.5^{a}	47.8±4.2^{b}
BUN (U/L)	9.7±0.9^{a}	11.2±1.2^{a,b}	11.6±1.8^{b}

Calcium (Ca) and Phosphate (PO_4^{3-}) in livers

There were increasing levels of Ca concentrations in the liver of meagre for fish fed the D1 and D2 diets, respectively, and a decrease for fish fed the D3 diet (**Figure 10**). However, significantly different values were found only between the D0 and D1 diets and the D0 and D3 ($P<0.05$). Internal high variation was indicated for the fish fed the D2 diet. An increase of phosphate level was indicated between D0 and D1 diets but it was not significant. Moreover, a decrease was evident for D2 and D3 diets compared to D0 and D1 but only the D2 diet differed significantly compared to the D0 and D1 diets ($P<0.05$).

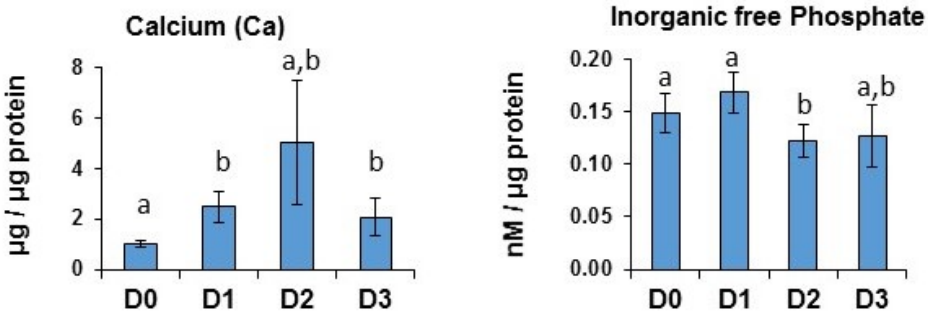

Figure 10. Concentrations of Ca and Phosphate in the liver of meagre. Fish were fed for three months with a control diet (D0) and diets supplemented with different levels of vitamin D_3 [D0=0.114 mg/kg (4550 IU), D1=0.175 mg/kg (7000 IU), D2=0.250 mg/kg (10000 IU), D3=0.500 mg/kg (20000 IU)], (n=9 fish/diet).

Antioxidant enzymes (SOD, CAT, GSH) in liver tissue

An increase in the antioxidant enzyme activities was revealed in all enzymes (SOD, CAT) and GSH content according to the increasing levels of vitamin D in the two diets D1 and D2, which was not significant except for the CAT activity and GSH content in fish fed D2 diet ($P<0.05$) (**Figure 11**). Moreover, SOD activity in fish fed the D_3 diet decreased in comparison to fish fed the D1 and D2 diets but this decrease was not significant. However, CAT activity and GSH content in fish fed D3 was reduced significantly in comparison to all other diets ($P<0.05$).

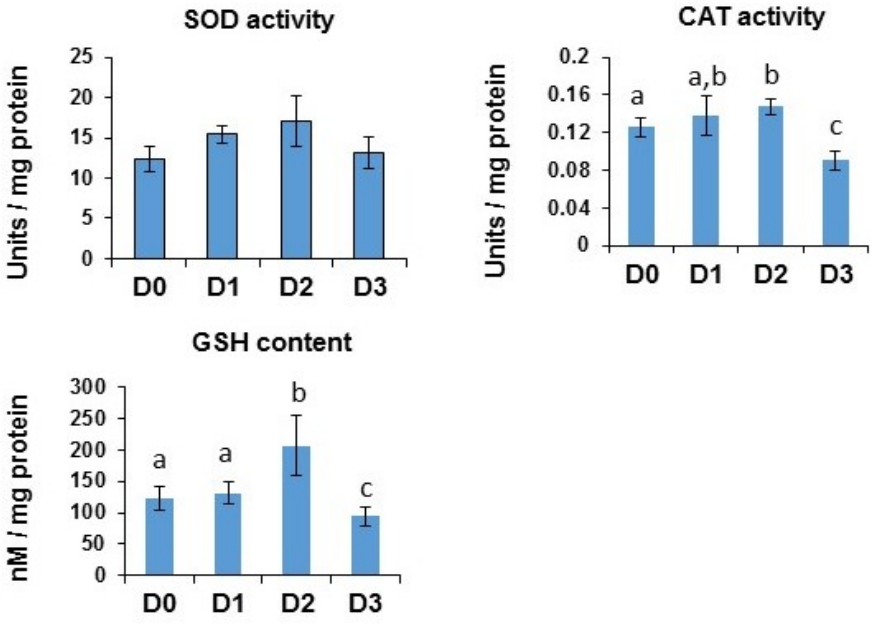

Figure 11. Activities of antioxidant enzymes in the liver of meagre. Fish were fed for three months with a control diet (D0) and diets supplemented with different levels of vitamin D_3 [D0=0.114 mg/kg (4550 IU), D1=0.175 mg/kg (7000 IU), D2=0.250 mg/kg (10000 IU), D3=0.500 mg/kg (20000 IU)], (n=9 fish/diet).

Vitamin D metabolizing enzymes (CYP27, CYP24)

Results on relative expression of CYP27A mRNA indicated down-regulation for fish fed the D1, D2 and D3 diet compared to the D0 diet (**Figure 12**) but significantly lower relative expression was indicated only for D1 and D2 diets in comparison to the other two diets (D0 and D3) ($P<0.05$). Pearson correlation two tailed analysis between the results of CYP27A mRNA and the percentage of the presence of granulomas in the liver of fish fed the diets with the different increasing levels of vitamin D_3 indicated a high negative correlation ($r = - 0.959$) and strong statistical significance ($p = 0.04$) (**Figure 13**). Where there was down-regulation of the relative expression of CYP27A there was an increase in the percentage of the presence of the granulomas, while where an up-regulation was indicated there was a reduction in the percentage of the presence of granulomas. Two tailed Pearson correlation analysis between calcium concentration and percentage of the presence of granulomas showed high positive correlation ($r = 0.929$) but weak statistical significance ($p = 0.071$) (**Figure 14**). The r^2 (0.86) explains the variability of granulomas due to the variability of calcium concentration. Pearson correlation two tailed analysis between expression of CYP27A and calcium concentration pointed to high negative correlation ($r = - 0.900$) but very weak statistical significance ($p = 0.1$) (**Figure 5**). The r^2 (0.81) explains the variability of calcium concentration to the variability of CYP27A.

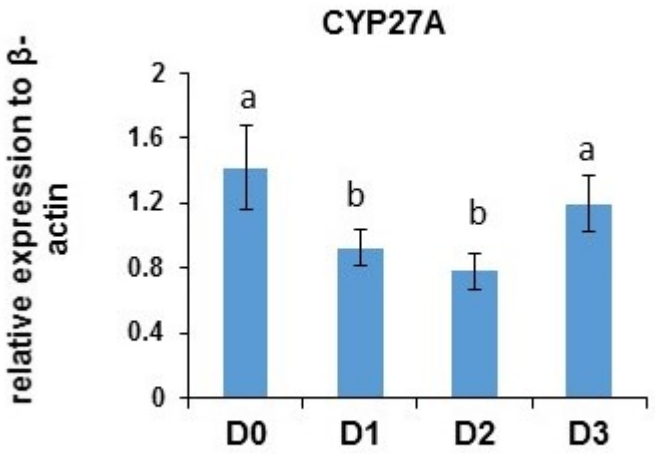

Figure 12. CYP27A relative expression levels to b-actin in liver of meagre. Fish were fed for three months a control diet (D0) and diets supplemented with different levels of vitamin D_3 [D0=0.114 mg/kg (4550 IU), D1=0.175 mg/kg (7000 IU), D2=0.250 mg/kg (10000 IU), D3=0.500 mg/kg (20000 IU)], (n=9 fish/diet).

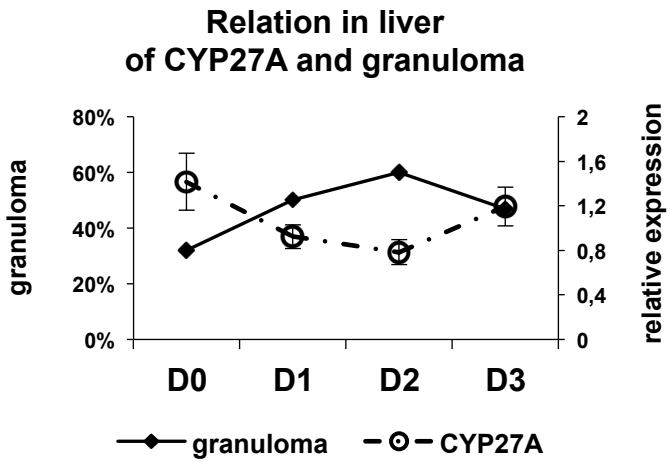

Figure 13. Comparative relation of CYP27A and granuloma presence (%) assessed in the liver of meagre. Fish were fed for three months a control diet (D0) and diets supplemented with different levels of vitamin D_3 [D0=0.114 mg/kg (4550 IU), D1=0.175 mg/kg (7000 IU), D2=0.250 mg/kg (10000 IU), D3=0.500 mg/kg (20000 IU)], (n=9 fish/diet).

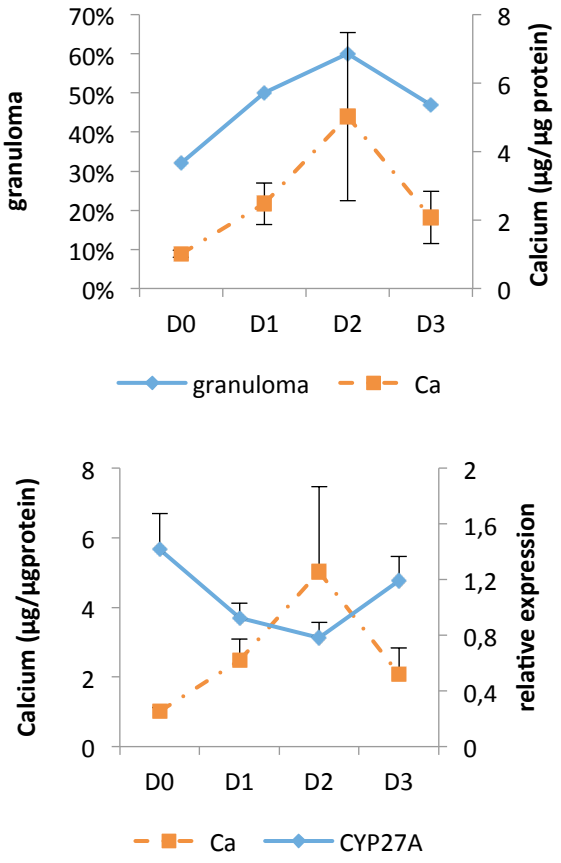

Figure 14. Comparative relation of Ca-granuloma presence (%) and Ca-CYP27A relative expression assessed in the liver of meagre. Fish were fed for three months a control diet (D0) and diets supplemented with different levels of vitamin D_3 [D0=0.114 mg/kg (4550 IU), D1=0.175 mg/kg (7000 IU), D2=0.250 mg/kg (10000 IU), D3=0.500 mg/kg (20000 IU)], (n=9 fish/diet).

Discussion

The development of SG was not prevented by vitamin D_3. The organs that seem to be affected first from SG were the kidney and the liver. The other soft tissues exhibited granulomas after the second month of the feeding trial, mainly visible by microscope.

No significant differences were recorded in growth parameters (weight, length, SGR, FCR) except in the condition factor (K) among fish fed different levels of vitamin D_3. The fish of the group D3 had the best condition factor. However, as long as no significant differences were recorded in weight and length of the fish, firm conclusions cannot be drawn from the increase in condition factor alone related to the increased dietary levels of vitamin D_3. Graff *et al*. (2002) also did not observe significant differences in fish weight or length between Atlantic salmon (*Salmo salar*) fed low, medium or high vitamin D_3. Similarly, Horvli *et al*. (1998) on Atlantic salmon smolt and Brown & Robinson (1992) on channel catfish (*Ictalurus punctatus*) reported normal growth rates when fish were fed large amounts of dietary vitamin D_3. In contrast, Andrews *et al*. (1980) in catfish and Vielma *et al*. (1998) in rainbow trout (*Oncorhynchus mykiss*) reported inhibition of growth performance due to high dietary vitamin D_3 intake.

Histological assessment confirmed the results of the visual inspection performed in fresh preparations. There was a clear chronic inflammation manifested with the granulomatous lesions, however there was also an implication of the vascular system. In many cases (not only observed in this experiment), the granulomas of meagre seemed to be of an infectious origin and had resemblance to the granulomas observed in mycobacterial infections such as the caseous necrotic areas. However, the failure of demonstrating infectious agents in histological sections using various staining techniques, such as Ziehl-Neelsen and Giemsa, or the negative results of bacterial cultures in various microbiological media (data not included in this deliverable) and the negative PCR results against various possible microbial agents (Nocardia, Mycobacteria, fungi etc) enforce the hypothesis of a metabolic disorder. In human medicine there are several non-infectious granulomatous diseases, metabolic and autoimmune, the pathogenesis of which is still unresolved. The implication of the vascular inflammation resembles Granulomatosis with Polyangiitis (formerly Wegener's granulomatosis), which is also a human autoimmune disease of unknown aetiology. Another interesting finding is the involvement of the rodlet cells in Systemic Granulomatosis. Rodlet cells are enigmatic cells that are unique in fish. For many years they were considered myxosporean parasites, however molecular analysis has proven that these are endogenous cells possibly inflammatory. The distinctive characteristics of these pear-shaped cells are the collection of the rodlets (linear crystal structures) within their cytoplasm and the thick surrounding membrane. Under specific conditions rodlet cells expel their rodlets into the extracellular environment (DePasquale, 2014). The composition of the rodlets is not known, however it has been shown that they contain the antimicrobial peptide piscidin (Silphaduang *et al*., 2006). Thus, their secretory nature might be connected to the defense mechanism of meagre against infection. However, this cannot be fully supported since there is no data on the presence of these cells in normal or wild specimens.

Measurement of plasma constituent levels can be an important indicator of the nutritional, physiological and clinical status of the fish. The advantage of this analysis is that it is easy to measure several samples in a short period using commercial kits, and also it can be carried out without killing the animal (Maita, 2007). However, the diagnostic value of this examination is

questionable, especially in fishes, due to the lack of reliable references of the normal values. Various factors such as age, sex, water quality, temperature and handling methods may contribute to variability in haematological data, thus it is difficult to compare results from different studies or set normal ranges (Maita, 2007; Tavares-Dias and Moares, 2007). Since data from wild meagre do not exist the observed values can be only compared to the values of other species. **Table 6** shows a compilation of data on plasma parameters in different fish species.

Regarding the results presented here, there were no significant differences in hematological indicators due to the differences in dietary vitamin D_3 except glucose levels, which were significantly higher in fish fed the lowest dose of vitamin D_3. In humans but also in mammals deficiency in vitamin D_3 impairs glucose tolerance and our result is probably related to this fact (Nazarian *et al.* 2011). In contrast to our results showing that serum calcium shows no difference among the diets, many authors report hypercalcaemic effects of vitamin D_3. Swarup *et al*. (1984) showed that in the catfish (*Clarias batrachus*) hypercalcaemia depends on the type and concentration of the vitamin D_3 metabolite used. Similar effects have been found in freshwater mud eel (*Amphipnous cuchia*), American eel (*Anguilla rostrata*) and male common carp (*Cyprinus carpio*) (Srivastav 1983, Fenwick *et al.* 1984, Swarup *et al*. 1991), but not for rainbow trout fed excess amounts of vitamin D_3 (Hilton & Ferguson 1982). However, results from Rao & Raghuramulu (1999) showed that vitamin D_3 probably plays no role at all in calcium or phosphorus metabolism in tilapia (*Tilapia mossambica*). It seems that the effect of vitamin D_3 on plasma calcium differs between studies so it could be possible that the levels in fish are not only controlled by the vitamin D_3 system but also by other hormones such as stanniocalcin, calcitonin and prolactin (reviewed by Lock *et al*., 2010).

From the comparison of our results with values from other species (**Table 6**) it is worth noting the increased values of phosphorus. Fish must obtain phosphate via the diet as water phosphate levels are normally very low and direct uptake of phosphate from the water is likely insignificant. Responses to vitamin D_3 metabolites on plasma phosphate vary between species (Lock *et al*., 2010). Hyperphosphatemia was noticed among eels treated with vitamin D_3 with no change in calcium levels. These results suggest that vitamin D_3 mediates phosphate homeostasis in marine fish, which live in an environment rich in calcium and poor in phosphorus (MacIntyre *et al.* 1976). Also, daily injection of vitamin D_3 increased plasma phosphate in catfish (Swarup *et al.* 1984), American eel (Fenwick *et al.* 1984) and common carp (Swarup *et al*. 1991). However, since hyperphosphatemia was also observed in the D0 diet where vitamin D3 level was low, it is possible that this observation is related to the disease itself and warrants further investigation. In humans, hyperphosphatemia is usually observed with concurrent low levels of blood calcium. Under this condition ectopic calcification is usually observed in soft tissues due to precipitation of phosphate-calcium salts. High blood phosphates in humans may be due to (i) impaired renal phosphate excretion caused by renal diseases, but also by hypoparathyroidism or parathyroid suppression, or (ii) by massive extracellular fluid phosphate loads caused by metabolic acidosis and cellular injury/necrosis. In meagre, as stated earlier, the first organ affected was the kidney and it is extremely difficult to assess whether the high levels of blood phosphates were the result of the kidney damage or whether the high phosphate levels were a contributing factor to the manifestation of the lesions (*e.g.* phosphate salt precipitation in soft tissues).

The analysis of serum liver enzymes such as ALT, AST and ALP was carried out since these have been proposed to be the main biomarkers for liver diseases (Hall and Cash, 2012). In general, the elevation of ALT and AST concentrations may indicate hepato-cellular diseases,

while the elevation in ALP may indicate cholestatic diseases of the liver (Sahoo *et al.,* 2015). Our results showed that, regardless of the diets, ALP, ALT and AST activity increased in fish with granulomas or tissue calcification compared with fish without. Increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation, while elevated plasma ALP activity corresponds to an inflammatory reaction of the bile ducts (Maita, 1997). In damaged tissues, cell membranes become more permeable, releasing some enzymes into the blood and thus modifying normal plasma values. In fish, elevated plasma ALP and AST have been associated with liver or bone disorders (Peres *et al*., 2013, 2014), so those results may be associated with SG, but further investigation needs to be done.

In human medicine it is customary and useful to categorize liver diseases into three broad categories: Hepatocellular, in which primary injury is to the hepatocytes; cholestatic, in which primary injury is to the bile ducts; and infiltrative, in which the liver is invaded or replaced by non-hepatic substances, such as neoplasm or amyloid. The AST, ALT, and alkaline phosphatase tests are most useful to make the distinction between hepatocellular and cholestatic disease. When AST and ALT levels are higher than ALP, then the condition is rather hepatocellular whereas when ALP is higher than AST and ALT the condition is cholestatic. In meagre, ALP was higher than AST and many times higher than ALT suggesting a cholestatic condition of the liver.

The ratio of the serum activities of AST and ALT was first described by Fernando De Ritis in 1957. The AST/ALT ratio became a useful indicator of the aetiology of hepatitis and it can be more clinically valid than assessing individual concentration (Botros and Sikaris, 2013, Sahoo *et al*., 2015). All the ratios of AST/ALT revealed from the results of this trial were>20 which suggests severe hepatic dysfunction was probably related to both SG and lipoid liver degeneration, a condition common in farmed fish.

Furthermore, our results showed that BUN concentration and LDH activity increased in fish with tissue calcification compared with fish without. BUN is a waste product produced from the breakdown of protein in the liver and it is usually eliminated from the body by the kidneys. So, both the livers and the kidneys must be functioning properly for the body to maintain a normal level of urea in the blood (Ajeniyi and Solomon, 2014). LDH is considered an indicator of tissue damage owing to pathological processes, toxic chemical exposure, or traumatic fish handling. Increased plasma LHD may indicate renal failure, muscle tissue breakdown, hemolysis, stress induced by handling or hypoxia (Peres *et al*., 2013). Therefore, the increased levels of those two parameters are probably related to the necrotic calcified tissues of liver and kidney.

In contrast, plasma total proteins decreased in fish with tissue calcification compared with fish without calcified tissues. Total protein level is useful for diagnosis and treatment of a variety of diseases involving the liver, kidney, or bone marrow, as well as other metabolic or nutritional disorders. Liver is the organ that is responsible for producing protein, so any condition that affects the liver, such as hepatitis, will cause the protein level in the blood to decrease.

The increasing activities of antioxidant enzymes recorded in fish fed the D1 (7000 IU) and D2 (10000 IU) diets confirm the action of supplemented vitamin D_3 as an antioxidant. However, at high dose (20000 IU) supplementation of D_3 suppresses antioxidant enzyme activities. In this case we cannot exclude the possibility that high doses of vitamin D_3 may produce oxidative stress to meagre. These outcomes can be compared to that of mammals when an increase in the amount of the intermediate and final lipid peroxidation (LPO) products has

been found in the vessels of rabbits injected with high dose of D_3 (10.000 IU/kg, hypervitaminosis), whereas a decrease in the antioxidant enzyme activities of SOD, GPx and CAT has been revealed (Harbuzova 2002). In humans with inflammatory disease supplementation of 1600 IU vitamin D_3 increased erythrocyte SOD and CAT activities, while SOD activities were positively correlated with serum 25(OH)D_3 levels (Javanbakht *et al*., 2010).

To our knowledge the present study is the first trial where increased vitamin D_3 dietary levels were associated with changes in metabolizing enzymes of vitamin D_3, and particularly to the first hydroxylation, with the SG in meagre. Our results revealed that there is a high negative correlation of sterol 27-hydroxylase (CYP27A1), which carries out the 25-hydroxylation of vitamin D_3, with the percentage of the presence of granulomas in the liver of meagre. Moreover, a high positive and a high negative association were revealed between calcium and percentage of granulomas and calcium and relative expression of CYP27A, respectively. However, these outcomes do not imply that the cause for granulomas in the liver of meagre is the level of vitamin D_3. However, it is possible that the levels of the different vitamin D_3 metabolites may play a crucial role in calcium and D_3 metabolism in SG. For example, in mammals, overproduction of the active metabolite of vitamin D_3 1,25-dihydroxyvitamin D3 (1,25(OH)2D) has been described in sarcoidosis and other granulomatous diseases. High circulating concentrations of 1,25(OH)2D lead to increased intestinal absorption of calcium, possibly to enhance bone resorption, and may result in hypercalcaemia and/or hypercalciuria. Data obtained in vivo and in vitro demonstrated that the unregulated production of 1,25(OH)2D lies within the granulomatous tissue and is controlled by glucocorticoids (Fuss *et al*., 1992). Further research must include, among other things, the evaluation of vitamin D3 metabolites.

In conclusion the addition of vitamin D_3 did not prevent the development of SG in meagre. However, the study provided a significant lead concerning the pathophysiology of SG that will further assist the detailed description of this peculiar disease.

Table 6. Plasma biochemical parameters in meagre fed four diets with 4550 (D0), 7000 (D1), 10000 (D2) and 20000 (D3) IU/Kg vitamin D, in meagre with no granulomas (0), granulomas (1) and calcification of at least one tissue (5), in humans and in different fish species.

	A.regius				A.regius			Human	Seabass	Seabream	Seabass	Oyster toadfish	Striped Seabream	Common Dentex	Gilthead Seabream	Acantopagrus latus	Epinephelus coioides	Rainbow trout
	D0	D1	D2	D3	0	1	5		Coz-Rakovac et al., 2005	Peres et al., 2013	Peres et al., 2014	Mensinger et al., 2005	Yildiz, 2009			Akbary P., 2014		Charoo et al., 2015
ALP (U/L)	139,5±14,7	139,0±24,8	124±13,3	135,8±14,5	120,3±8,9	147,5±15,3	136,0±12,5	50-160	-	123,1	51,1	123,2±9,7	-	-	-	-	-	73
AST (U/L)	96,7±8,1	81,8±12,9	81,2±7,4	100,2±24,3	77,3±9,2	93,4±7,3	99,3±19,8	0-40	44,0	64,0	83,6	41,1±5,2	-	-	-	-	-	195
ALT (U/L)	1,7±2,4	2,3±3,3	2,5±1,8	3,5±2,0	0,1±1,3	2,6±0,6	4,8±1,0	0-30	<5	-	-	19,1±1,8	-	-	-	-	-	-
BUN (U/L)	10±0,7	11,0±0,9	11,5±2,4	10,9±0,7	9,7±0,7	11,2±0,9	11,6±1,5	20-40	-	-	-	5,8±0,5	-	-	-	3,82±0,55	2,66±0,45	-
Ca (mmol/l)	1,6±0,4	1,7±0,5	1,5±0,2	1,3±0,2	1,4±0,2	1,5±0,3	1,8±0,4	2,25-2,75	-	3,8	4,0	2,3±0,05	2,65±0,11	3,49±0,11	2,82±0,09	19,80±2,97	16,80±2,34	9,98
P (mmol/l)	8,7±0,8	8,0±0,5	8,2±1,1	9,3±0,5	9,0±0,6	8,8±1,0	7,9±0,6	0,97-1,45	-	3,7	2,7	-	3,08±0,12	4,56±0,21	2,90±0,08	2,35±0,40	2,40±0,57	2,87
Mg (mmol/l)	0,8±0,0	0,8±0,1	0,8±0,1	0,7±0,1	0,7±0,1	0,8±0,0	0,8±0,1	0,73-1,48	-	1,2	1,5	-	-	-	-	-	-	2
Trig (mg/dl)	301,3±11,1	283,0±34	307,0±24,4	270,3±38,6	274,1±34,7	303,3±21,2	293,9±29,2	4-200	59,3	289,1	405,5	49,1±4,8	-	-	-	102,02±11,82	68,10±15,34	-
LDH (U/L)	72,1±21,2	62,7±8,0	59,4±2,0	55,4±9,7	53,3±5,2	63,0±12,8	70,8±12,8	105-333	-	618,5	126,6	-	-	-	-	-	-	-
CHOL (mmol/l)	3,3±0,5	3,6±0,5	3,0±0,2	3,3±1,0	3,0±0,7	3,5±0,5	3,4±0,5	<5,83	2,7	9,4	7,1	3,40±0,20	-	-	-	6,66±0,92	3,56±1,1	3,83
Glu (mmol/l)	5,1±0,4	3,2±0,8	3,0±0,8	3,6±0,7	4,2±0,5	3,2±1,1	3,7±1,3	4,44-6,66	3,7	6,0	7,2	-	4,06±0,11	4,25±0,08	4,65±0,43	2,46±0,90	2,43±0,56	9,32
TP (g/l)	57,5±6,9	59,5±9,7	56,3±12,3	50,0±8,9	58,4±10,0	61,4±6,6	47,8±4,3	63-80	36,0	45,0	49,0	49±5	-	-	-	43,1±3,2	39,2±3,6	33

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