First year fellowship results

Insulin resistant adipocytes obtained *in vitro* using a mouse cell line. During differentiation, 3T3-L1 cells acquire the capability to store triglycerides (TGs) and the adipocytes are lipid laden by day 9, showing a readily recognizable morphology. Mature adipocytes continue to store and increase their TG content and they appear hypertrophic at day 21. To assess the lipid accumulation, the adipocytes were stained with Oil red O at days 9 and 21 of the differentiation protocol. The accumulation of lipid droplets within the cells was markedly increased at day 21 when compared with the cells at day 9 (Figure 1A). Next, to assess insulin sensitivity as a functional aspect of the adipocytes, both western blot analysis and 2-deoxyglucose (2-DG) uptake assays were performed. As insulin resistance is associated with an impaired Akt activation, insulin-induced phosphorylation of Akt at Ser^{473} was detected using a phospho-Akt (Ser^{473}) antibody. A substantial decrease in insulin induced phosphorylation was observed in the adipocytes at day 21 compared with the cells at day 9. Equal loading was confirmed by similar concentrations of β-actin (Figure 1B). In addition, the adipocyte glucose incorporation in both basal and insulin stimulation conditions was evaluated by measuring the 2-[^{14}C]DG uptake in the absence of cold glucose in the medium. As shown in Figure 1C, the hypertrophic cells (3T3-L1 at day 21) had a considerably lower increase in glucose uptake under insulin stimulation when compared with the adipocytes at day 9. Indeed, the net insulin stimulated 2-DG uptake achieved the peak level of 5-fold over basal at day 9 and then decreased, as the cells became hypertrophic, to less than one-half of the peak value, suggesting that the hypertrophic adipocytes are resistant to insulin stimulation.

Furthermore, as an example of an obesity-associated miRNA to validate this system, the expression of miR-122 (miR-122-3p) was evaluated by real-time quantitative PCR assay (RT-qPCR). MiR-122 levels progressively increased during adipocyte differentiation (data not shown), reaching a maximum when the cells were certainly hypertrophic. On day 21, the expression of miR-122 was 2.5-fold higher than the expression of the same miRNA on day 9, thus demonstrating that miR-122 expression parallels lipid accumulation and the development of insulin resistance (Figure 1D). These data confirmed in this *in vitro* obese model, consisting of hypertrophic 3T3-L1 adipocytes, what had already been shown in rodent diet-induced obesity models and in obese patients in the clinic[37-40].

Afterwards, the serum-free conditioned media (CM) were harvested from the 3T3-L1 adipocytes at days 9 and 21 of the differentiation protocol and stored at -80 °C until use.

Figure 1. Lipid accumulation and insulin sensitivity in adipocytes at days 9 and day 21 of differentiation. A. Adipocytes at day 9 and day 21 of differentiation stained with Oil red O. Representative photographs at a magnification of 10X (left panel) and 20X (right panel) are shown. **B1**. Western blot analysis performed by separating adipocyte cell lysates (30 µg of protein per lane) on an SDS/PAGE gel (10%) and probing with an antibody against phospho-Akt (Ser^{473}). The immunoblot showed is representative of three independent experiments. **B2**. The bar graph represents the densitometric quantification of three immunoblots corresponding to three independent experiments. *** indicates a P value **C.** 2-DG uptake by the adipocytes at day 9 and day 21 of differentiation in the absence (basal) or presence (stimulated) of insulin. Data represent mean values ± SD of triplicate samples of three independent experiments. *** indicates a P value <0.001. **D.** MiR-122 expression at day 9 and day 21 of adipocyte differentiation. The results are normalized to the expression of the RNA U6 Small Nuclear (RNU6 or U6*).* The real-time PCR data are presented as the fold change over the day 9 value. ** indicates a P value <0.01.

Figure 1.A

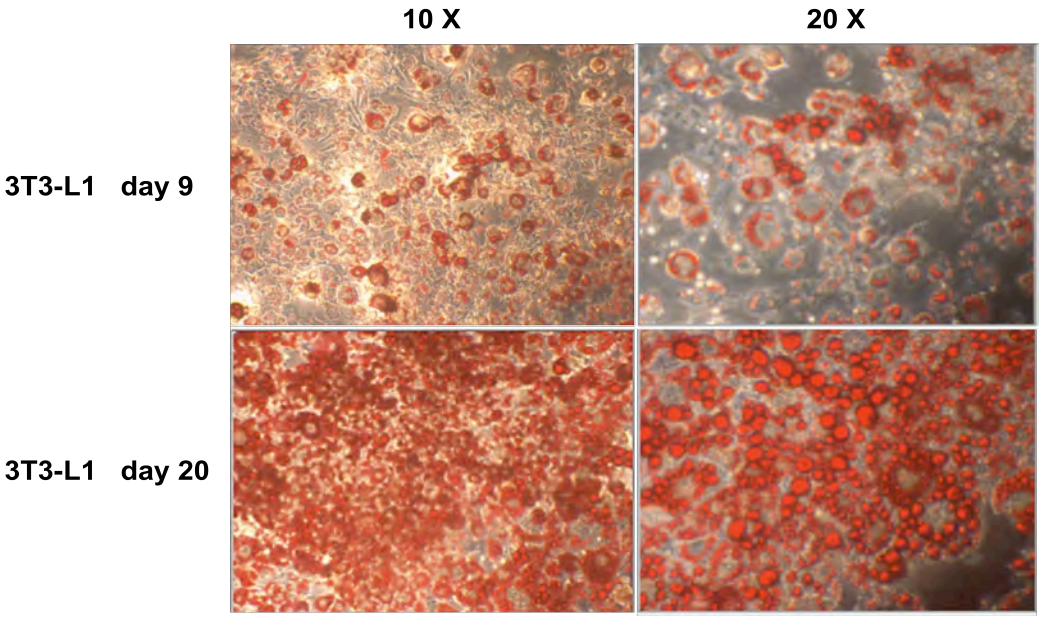


Figure 1.B1

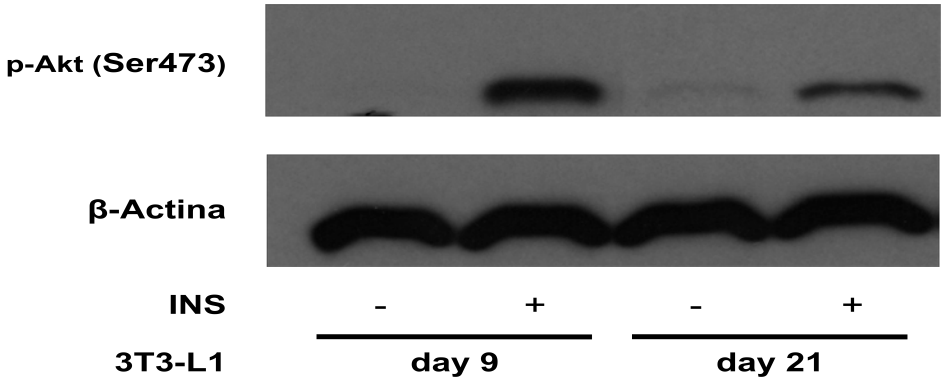


Figure 1.B2

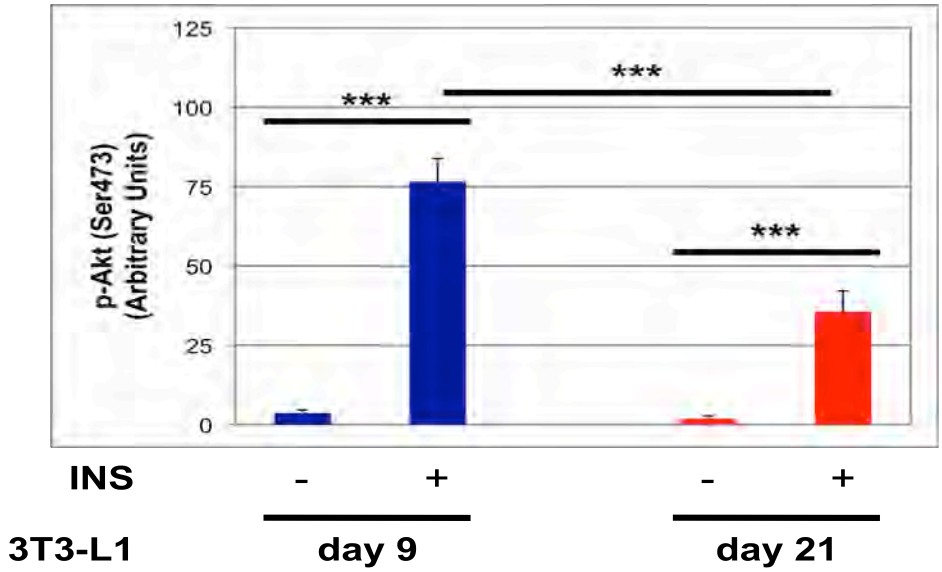


Figure 1.C

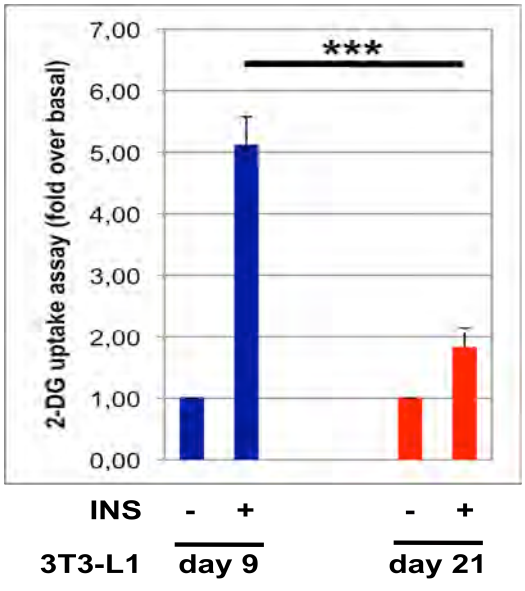
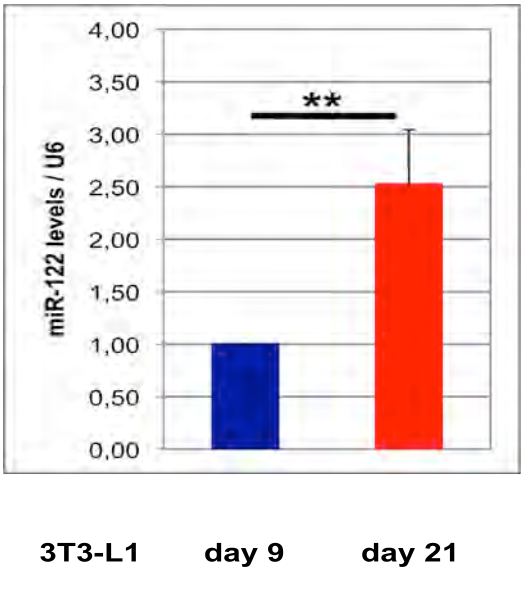


Figure 1.D



Insulin resistant adipocytes obtained *ex vivo* from a mouse model of obesity and diabetes. 9 male C57BL/6 mice were fed a high fat diet (HFD; 60% calories from fat) for 12 weeks to induce obesity, while 9 male C57BL/6 mice were fed a standard diet as lean controls. At the end of the diet period, insulin resistance was observed in the obese mice. The characterization of these mice is shown in the following table:

Table 1.

parameters	lean mice (*n* = 9)	obese mice (*n* = 9)	T test
Body Weight (g)	31.6 ± 3.3	42.3 ± 6.3	P<0.0001
Fasting Blood Glucose (mg/dl)	105.8 ± 18.9	158 ± 37.51	P=0.0002
* GTT [AUC] (mg/dl·120 min)	8990 ± 1586.5	17892 ± 8418.8	P<0.0001
** ITT [AUC] (mg/dl·120 min)	9020 ± 3993.5	13977 ± 2839.6	P=0.0003

* glucose tolerance test (GTT) - area under the curve (AUC)

** insulin tolerance test (ITT) - area under the curve (AUC)

According to the values reported in Table 1, the HFD resulted in an increased body weight gain and a significant increment in plasma glucose concentration above the baseline. Under the HFD, the efficiency of the insulin was reduced in insulin tolerance tests (ITTs), demonstrating a decrease in insulin sensitivity for the obese mice; in addition, glucose tolerance tests (GTTs) revealed that the obese mice had a significantly worse glucose tolerance than the lean controls.

The mice were then sacrificed and, in addition to collecting adipose tissue, skeletal muscle and sera for further RNA and protein isolation, epididymal adipose tissue from each mouse was immediately minced into pieces and digested to separate the floating population of mature adipocytes from the stromal vascular fraction pellet. Afterwards, the serum-free conditioned media (CM) were harvested from the mature adipocytes of both the obese and lean mice and stored at -80 °C until use.

Insulin resistant myotubes obtained upon treatment with the conditioned media of insulin resistant adipocytes. Adipocyte CM were used for 24-hour treatments in L6 myotubes, the best-characterized cellular model of skeletal muscle origin to study glucose uptake. Adipocyte CM from both 3T3-L1 cells and mice were used to treat L6 myotubes and the insulin-induced glucose uptake in the treated L6 myotubes was measured by 2-[^{14}C]DG in 2-DG uptake assay.

The glucose uptake was 1.98±0.27-fold over basal in the L6 myotubes treated with the CM from 3T3-L1 at day 9, while it was only 1.22±0.28-fold over basal in the L6 myotubes treated with the CM from 3T3-L1 at day 21. Since the glucose uptake was 1.60±0.23-fold over basal in the L6 cultured in DMEM (control cells), an improvement in insulin sensitivity was observed in the case of the CM from 3T3-L1 at day 9, while the CM from 3T3-L1 at day 21 caused an impairment, providing evidence of the acquired insulin resistance in L6 myotubes.

In the case of the L6 myotubes treated with the adipocyte CM from mice, the glucose uptake was 2.37±0.17-fold over basal in the L6 myotubes treated with the lean adipocyte CM, while it was 1.39±0.31-fold over basal in the L6 myotubes treated with the obese adipocyte CM. Therefore, an increased insulin sensitivity was demonstrated in the L6 myotubes upon treatment with the lean adipocyte CM, while the treatment with the obese adipocyte CM caused a significant decrease, considering that the glucose uptake was 1.78±0.23-fold over basal in the control cells cultured in DMEM.

Figure 2. Glucose uptake in myotubes. A. 2-DG uptake by L6 myotubes cultured in DMEM (green bars) or treated with the CM from adipocytes at day 9 and day 21 of differentiation in the absence (basal) or presence (stimulated) of insulin. **B.** 2-DG uptake by L6 myotubes cultured in DMEM (green bars) or treated with the CM from adipocytes of lean and obese mice in the absence (basal) or presence (stimulated) of insulin. The data represent mean values ± SD of triplicate samples of three independent experiments. * indicates a P value <0.05; ** indicates a P value <0.01; *** indicates a P value <0.001.

Figure 2.A

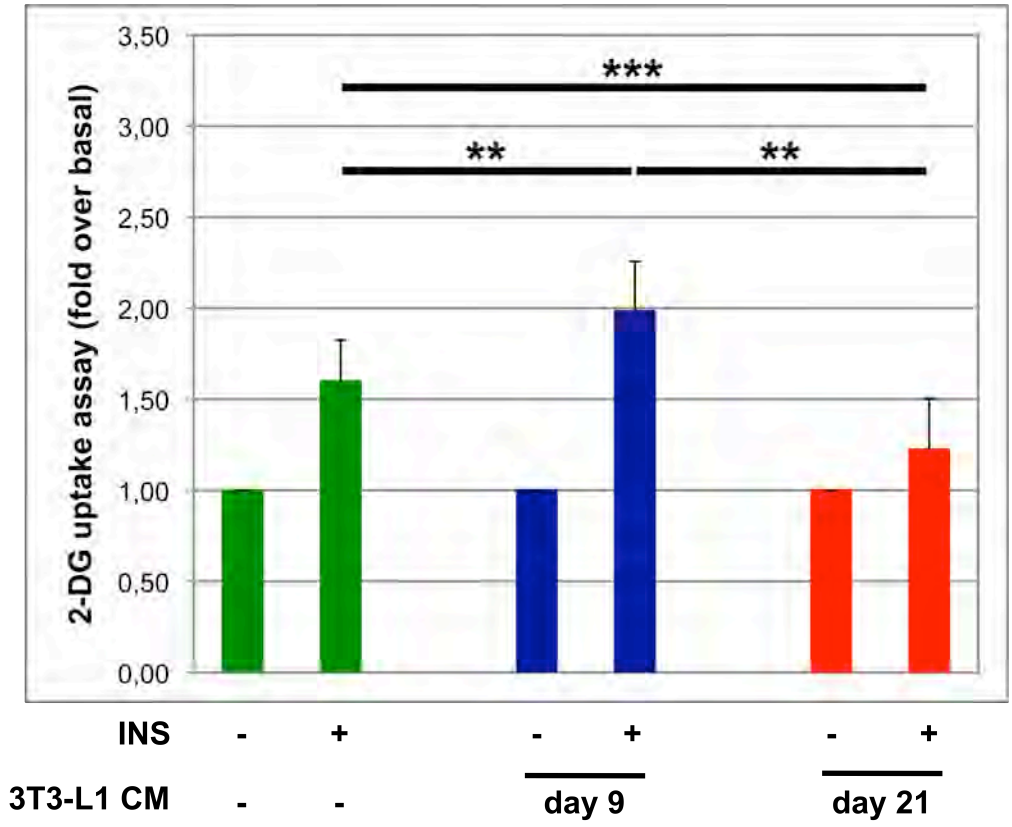
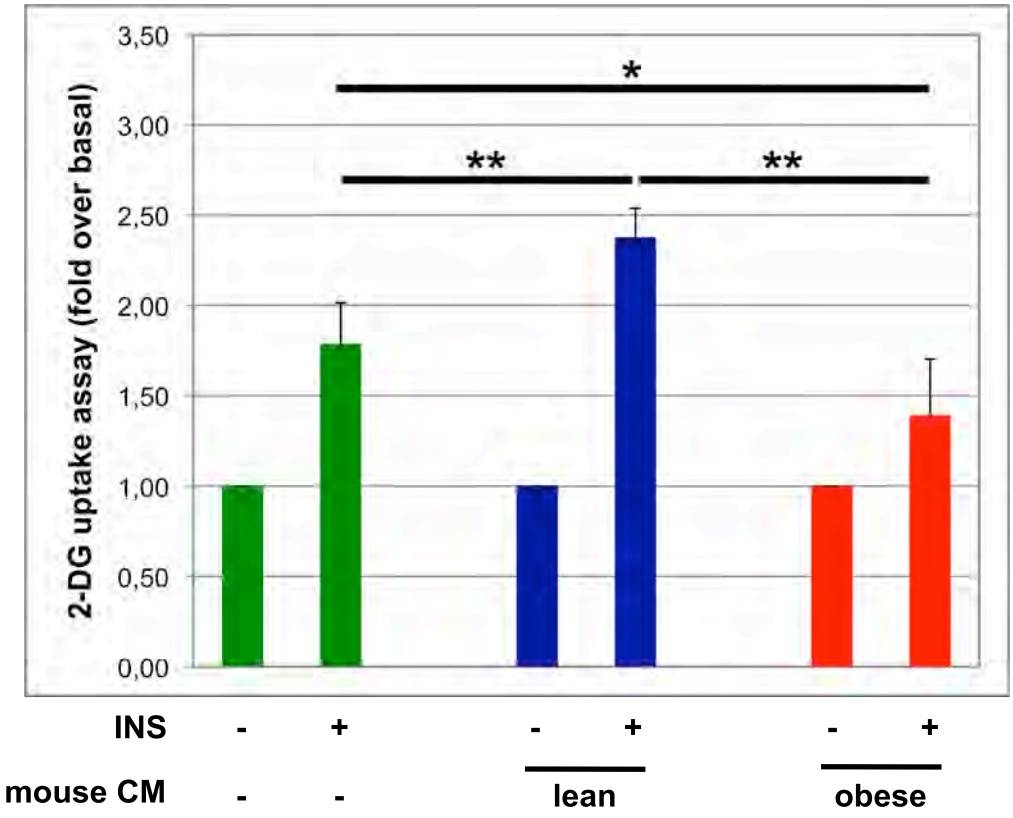


Figure 2.B



MiRNA profiling of myotubes treated with adipocyte conditioned media. Microarray-based miRNA profiling analysis was carried out for the L6 myotubes treated with adipocyte CM from 3T3-L1 cells using the Agilent miRNA microarray platform. Several differentially expressed miRNAs were identified in the L6 myotubes treated with both adipocyte 3T3-L1-day 9 CM (3 miRNAs up-regulated, Figure 3.A) and adipocyte 3T3-L1-day 21 CM (4 miRNAs up-regulated and 3 down-regulated, Figure 3.B) when compared with the expression patterns of the L6 myotubes cultured in DMEM. Standard selection criteria to identify differentially expressed miRNAs was established at 2-fold change in expression which corresponds to a log2 value of > +1 or < -1 and P-value < 0.05.

Figure 3.A

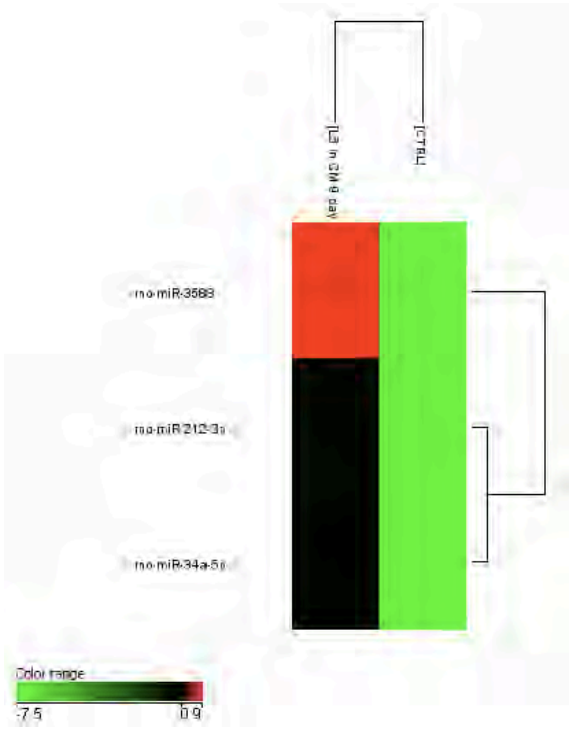


Figure 3.B

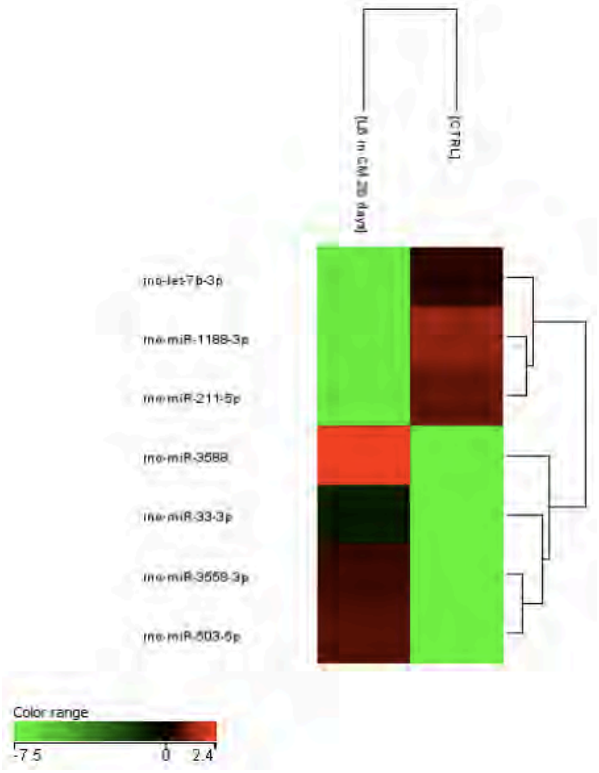


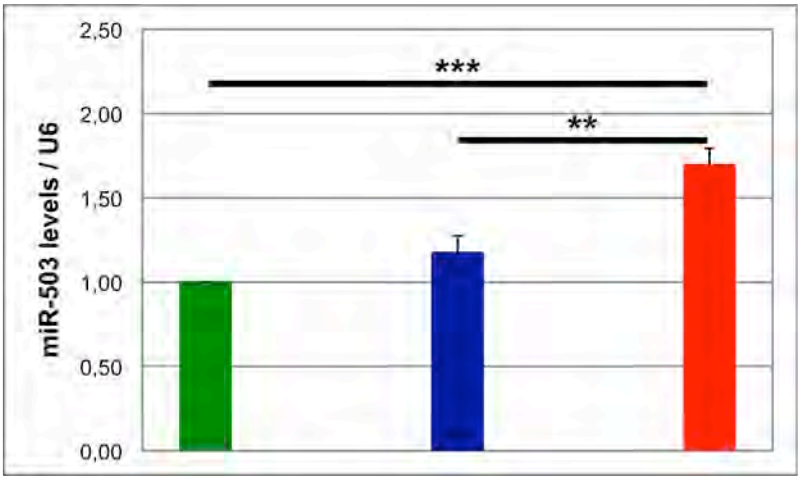
Figure 3. Microarray of L6 myotubes treated with adipocite 3T3-L1 conditioned media. MicroArray Hierarchical Clustering of L6 myotubes treated with the CM from adipocytes at day 9 vs L6 myotubes cultured in DMEM (**A**) and L6 myotubes treated with the CM from adipocytes at day 21 vs L6 myotubes cultured in DMEM (**B**). The Microarray experiment was conducted in three technical replicates for each sample.

Alternatively, miRNA sequencing (miRNA-seq) was chosen to analyze the miRNA expression profiling in the L6 myotubes treated with adipocyte CM from lean and obese mice. Compared with the L6 myotubes cultured in DMEM, the L6 myotubes treated with both the lean adipocyte and obese adipocyte CM showed several differentially expressed miRNAs. In the case of the lean adipocyte CM, 13 miRNAs were up-regulated and 8 down-regulated, while 16 miRNAs were up-regulated and 9 down-regulated in the obese adipocyte CM condition.

Next, the expression of individual miRNAs will be measured by the RT-qPCR method in order to verify the accuracy of the microarray and miRNA-seq results, since RT-qPCR is the most sensitive and reproducible method to quantify gene expression. Beginning with miRNA microarray expression data, the increase in miR-503 levels has been already validated in the insulin resistant L6 myotubes compared to the control L6 myotubes (1.69±0.10). miR-503 has been chosen first as already demonstrated to be involved in diabetes and its complications. In addition, it might regulate several targets in both the PI3K/AKT signaling pathway and GLUT4 translocation to the plasma membrane, according to the computational miRNA target prediction analysis I have also performed. Indeed, in order to better understand the biological function of this miRNA, its target genes were predicted using Targetscan and further classified to identify pathways that were actively regulated by miR-503. It is worth noting that 35 targets of miR-503 belong to the insulin signaling pathway, as shown by DAVID KEGG analysis.

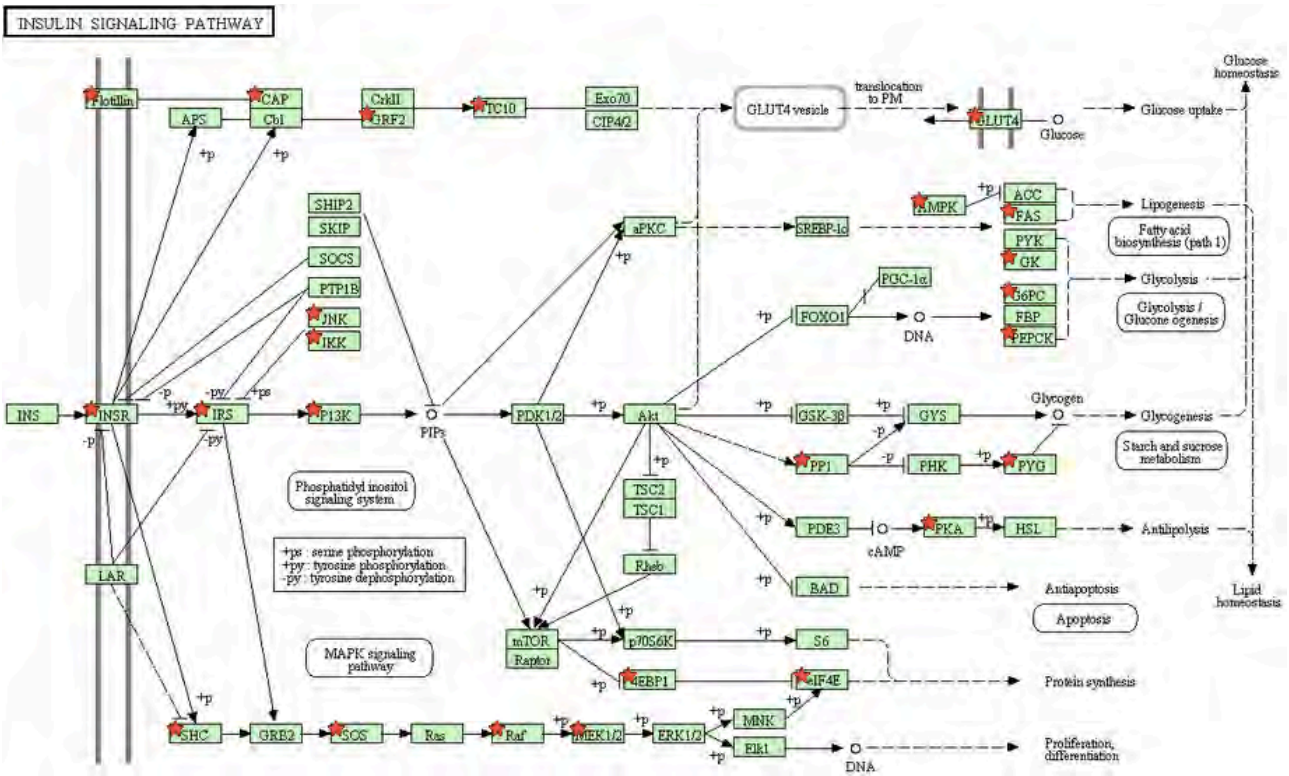
Figure 4. Validation for Microarray data of L6 myotubes treated with adipocite 3T3-L1 conditioned media. A. MiR-503 expression was evaluated by qRT-PCR in the L6 myotubes cultured in DMEM (green bar) and L6 myotubes treated with the CM from adipocytes at day 9 and day 21 of differentiation. The results were normalized to the expression of the RNA U6 Small Nuclear (RNU6 or U6). The real-time PCR data represent mean values ± SD of more than three independent experiments. The data are presented as the fold change over the DMEM value. ** indicates a P value <0.01; *** indicates a P value <0.001. (**B**). The putative targets of miR-503 participating in the insulin signaling pathway via DAVID KEGG analysis are marked with a red star.

Figure 4.A



3T3-L1 CM - day 9 day 21

Figure 4.B



Troubleshooting

As is reported in the previous project I sent you last year, I initially planned to use a mouse model of obesity and diabetes. For this reason, 9 mice were fed a high-fat diet of 60% cals from fat for 12 weeks in order to gain weight. At the end of the 12-week period, metabolic tests were performed on each obese mouse, together with 9 lean mice as controls, and insulin resistance was observed, as Table 1 shows in the result section. Next, both the obese and lean mice were sacrificed and several tissues were collected from each mouse and stored at -80 °C. Epydidimal adipose tissues were also collected, cut in small pieces and subjected to digestion by collagenase to obtain the various cellular components in distinct fractions. Mature adipocytes were incubated in serum-depleted DMEM/F12 with 0.1% (w/v) fatty acid-free BSA for 24 hours in order to obtain the conditioned media to use for the L6 myotube treatments. As the preparation of the conditioned media from the mature adipocytes was very time-consuming, I realized that it was better to have an easy and more manageable *in vitro* system of obese adipocytes. After conducting a detailed study of literature and making several attempts, I obtained 21-day old 3T3-L1 adipocytes, which were demonstrated to be hypertrophic by Oil red O staining and insulin resistant by western blot and 2-DG uptake assay (Figure 1). This unplanned task distracted me from all the other experiments I had arranged for the past year, even if it was not wasted time. Indeed, because of this new *in vitro* system of obese adipocytes, I am now able to prepare adipocyte conditioned media every time I need it and in a reasonably short time frame. My future work will undoubtedly gain improved outcomes and benefits using this *in vitro* system.

Another reason why I am now behind schedule was the time required for the high-throughput experiments and bioinformatic analysis in order to determine the miRNA expression profilings of the L6 myotube samples. As I asked for services external to our laboratory and Institute, I was not able to provide an accurate estimate of the time demanded for this task. Even if it has now successfully completed, I have to state that the work took more time than I had expected.

Concluding remarks

Although much work remains to be done on this project, I am confident that I will make good progress and achieve successful results in the next year. There are several reasons why a positive attitude towards my work should be adopted: firstly, in addition to the *in vitro* system available at any time to prepare fresh adipocyte conditioned media and promptly perform new experiments in L6 myotubes, mouse tissues for the *in vivo* studies are now stored at -80 °C and ready to use. Secondly, for the remaining part of the project, I expect to perform cell and molecular biology experiments and I have gained valuable skills in this field through my past experiences. Furthermore, during the last year, I have already had the opportunity to work with miRNA target prediction programs and web-based servers integrating multiple established prediction programs, which are all freely available.

The identification of the miRNAs involved in obesity-induced insulin resistance in skeletal muscle and the transfer of miRNAs between the adipose tissue and skeletal muscle are the next steps I have to demonstrate. As several miRNAs have already been observed to be differentially expressed in both the adipose tissue and skeletal muscle of high fat diet-induced obese mice[32-36], the driving and innovative concept of this project is that the c-miRNAs are transferred from the adipose tissue to the skeletal muscle. At this final destination, they are responsible, at least in part, for the obesity-induced insulin resistance, so contributing to explain the causal link between obesity and insulin resistance. Thus, the results of this study might represent an encouraging starting point in the search for new approaches in what is called the fight against “globesity”. Indeed, as a future prospect in humans, the detection of the proposed miRNAs in serum/plasma might lead to an earlier diagnosis of diabetes linked to obesity. In addition, novel strategies aiming to restore the altered levels of miRNAs might be promising in the clinic as a potential new line of intervention to prevent or treat insulin resistance and diabetes associated with obesity.

Budget

A detailed total budget must be provided. The budget period (time) during which the grant funds was spent according to the specific needs of the project must be clearly stated and justified where indicated.

Total budget period (in months): 12 months (year 2015)

Supplies (description): RNA isolation kits gene expression reagents	**TOTAL Euro** 6000,00	**SID FO.DI.RI.** **to Claudia Miele**
Supplies (description): animal housing and care cell culture reagents chemicals antibodies for WB microRNA Microarray MicroRNA sequencing (miRNA-seq)	**TOTAL Euro** 18000,00	**MERIT RBNE08NKH7** **to Francesco Beguinot**
Personnel	**TOTAL Euro** 25000,00	**Fellowship to Paola Mirra** **by FONDAZIONE DIABETE RICERCA - MSD**
Consultant / contractual costs	**TOTAL Euro** 0,00	
	TOTAL COSTS Euro 49000,00	