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The insulin-mimetic effect of two Vanadium pyridinone complexes on human visceral adipose tissue

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Abstract

Diabetes Mellitus is a metabolic disease that affects a significant portion of the world's population. Disease management is mainly achieved through lifestyle change and medication. Despite great progress, it remains a challenge. In this context, there is an ongoing need to develop new therapeutic approaches.

Several Vanadium compounds have been tested through the years as possible antidiabetic drugs after the discovery of this effect in 1899. Initially research efforts focused on inorganic salts, which eventually proved to have unsafe therapeutic profiles. Nowadays, most efforts focus on organically chelated vanadium compounds, due to their wider therapeutic window.

In this work, VO(mpp)2 and VO(dmpp)2 were tested as to their influence on human adipose tissue metabolism, and the insulin signalling pathway. For this, human adipose tissue was collected from patients with obesity. Tissue was exposed to these compounds as compared to $VOSO_4$, a common reference compound, and a control, at three different concentrations in combination with insulin.

We studied the bioenergetic profile using a Seahorse analyser; GLUT4 translocation using immunostaining; and downstream insulin signalling by quantifying phospho-ERK load by western blot.

Both mitochondrial and non-mitochondrial oxygen consumption rates were increased across all vanadium experimental conditions, in particular in respect to $VOSO_4$. GLUT4 translocation with in the control stood out as stronger than all other conditions. Amongst vanadium compounds, VO(dmpp)2 showed higher membrane GLUT4 labelling. Phospho-ERK showed inconsistent results throughout the studied conditions.

In conclusion, our results point to increased oxidative stress in adipose tissue both with the traditional inorganic salt and the more recent oxovanadium complexes, while a specifc effect on insulin signalling could not be attested. Future studies, with larger sample sizes are needed to further validate results.

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Table of Contents

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Gaudeamus igitur, iuvenes dum sumus!

III. List of Abbreviations

IDDM – Insulin-Dependent Diabetes Mellitus

NIDDM – Non-Insulin-Dependent Diabetes Mellitus

IR – Insulin Receptor

IRS-1 – Insulin Receptor Substrate 1

PI3K – Phosphoinositide 3-Kinase

PIP2 – Phosphatidylinositol Biphosphate

PIP3 – Phosphatidylinositol Triphosphate

PDK1 – Phosphatidylinositol-Dependent Kinase

PKB – Protein Kinase B

GS – Glycogen Synthase

PTP-1B – Protein Tyrosine Phosphatase 1b

V – Vanadium

V_2O_5 – Vanadium Pentoxide

PLGA – Poly-Lactoglycolic Acid

ROS – Reactive Oxygen Species

BMOV – Bis(Maltolato)Oxovanadium

$VOSO_4$ – Vanadyl Sulfate

Na_3VO_4 – Sodium Orthovanadate

$NaVO_3$ – Sodium Metavanadate

STZ – Streptozotocin-Treated

BEOV – Bis(Ethylmaltolato)Oxovanadium

IGF-IR – Insulin-Like Growth Factor Receptor

pp – 3-hydroxi-4-pyridinone

pic – Picolinate

dipic – Dipicolinate

Xmpic – Methylpicolinates

5Ipic – Iodopicolinate

4Clpic – Chloropicolinate

FFA – Free Fatty Acid

DMSO – Dimethyl Sulfoxide

CHEDV – Centro Hospitalar entre Douro e Vouga

VAT – Visceral Adipose Tissue

DMEM/F12 – Dulbecco's modified Eagle medium: Nutrient Mixture F-12

FCCP – Carbonyl Cyanide-P-Trifluoromethoxyphenylhydrazone

OCR – Oxygen Consumption Rate

RELi – Removal of Excess Lipids

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM – Standard Error of the Mean

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1. Introduction

1.1. Diabetes and Insulin resistance

It is estimated that up to 10% of the worlds population is currently suffering from Diabetes Mellitus and that the worldwide number of diabetics could rise to 366 Million by 2030. (Li & Lian, 2016) It is a metabolic disease mainly characterized by highly volatile blood glycaemia levels, with differing variation profiles according to the type of diabetes, which consequently leads to disruption in carbohydrate, protein and lipid metabolism. As it is so disruptive of anabolic and catabolic processes it has wide systemic health effects, not just limited to the daily dangers of glycaemic fluctuations, but also greatly increasing the risk for several acute and chronic ailments such as stroke, peripheral neuropathy and arterial disease, cardiomyopathy, nephropathy, retinopathy, immune system dysfunctions with increased risk for infections and poorer outcomes, deficiencies in wound healing, diabetic foot ulcer, several oncologic diseases such as pancreatic adenocarcinoma and many others. (Li & Lian, 2016)

There are two main types of diabetes that account for the large majority of cases, type I and II. Type I, or insulin-dependent diabetes mellitus (IDDM), accounts for 10% of cases worldwide and is caused by the inability of the pancreas to produce sufficient, if any, quantities of insulin. (Maraschin, 2012) The beta cells in the Langerhans' islets of the pancreas are destroyed or disrupted by an autoimmune response or trauma and, thus, even though the body's cells react appropriately to insulin signalling, exogenous insulin must be periodically administered. This form commonly first manifests at a young age. (Maraschin, 2012)

Type II, non-insulin-dependent diabetes mellitus (NIDDM), is the vastly more prevalent form (90% of diabetes cases). Contrary to type I it is a downstream problem, whereby insulin production is not the triggering factor, the tissue cells' signal response is. (Maraschin, 2012) The insulin receptor (IR) signalling pathway has a diminished sensitivity to insulin, which results in, among other things, less translocation of the GLUT4 glucose transporter vesicles to the cellular membrane, decreasing the cell's glucose uptake capacity. This recurrently causes hyperglycaemia, prompting beta cells to produce more insulin until the new signal threshold is met and blood glucose decreases effectively. Over time this feedback loop leads to hyperinsulinemia and increasing insulin resistance. It is most prevalent among older adults and the elderly and is strongly associated with excessive body weight and obesity, dietary over-consumption of sugars and saturated fats, highly sedentary lifestyles and tabagism. (Domingo & Gómez, 2016; Maraschin, 2012)

Disease management is essentially accomplished by lifestyle and dietary adjustment and pharmaceutical therapy. Some oral medications like metformin are used for this effect, but have limited effects. Insulin injection is currently the most effective way of controlling glycaemia and exists in fast, medium and slow acting forms. (Laws et al., 2012) Insulin has a very low

bioavailability in oral administration, thus, patients need regular injections, often self-administered, and this causes discomfort, decreased therapeutic compliance, and increased risk of injection site reactions. This, together with the fact that long-term insulin administration may increase tolerance, is the main reason that oral alternatives, such as in the case of vanadium compounds, have been so researched. (Domingo & Gómez, 2016; Li & Lian, 2016)

1.1.1. The role of Insulin

Insulin is a fundamental hormone as it regulates glucose uptake in the main anabolic centres of the body. When blood glucose levels rise after absorption of sugars through the intestinal tract, the beta cells in the pancreas increase their secretion of insulin to signal this availability. (Sonksen & Sonksen, 2000) Hepatocytes, adipocytes and striated muscle cells, cardiac and skeletal, all have membrane insulin receptors which initiate a signalling cascade culminating in the translocation and fusion of glucose transporter GLUT4 intracellular vesicles to the cell membrane, greatly increasing the diffusion of glucose to the cell, and also in the inhibition of catabolic processes and promotion of anabolic ones. By this effect there is an upregulation of protein synthesis and glycogenesis in striated muscle cells, lipogenesis in adipocytes and of all of these in hepatocytes, and a strong downregulation of gluconeogenesis in hepatocytes. (Sonksen & Sonksen, 2000; Weiss et al., 2000)

Upon binding to the insulin receptor alpha units, autophosphorylation of the beta units tyrosine residues occurs, this allows for the binding of the insulin receptor substrate (IRS-1) which is phosphorylated – this IRS-1 mediation is the main insulin signal transduction pathway. IRS-1 then binds to the p85 subunit of phosphoinositide 3-kinase (PI3K), activating it, causing its catalytic p110 subunit to phosphorylate phosphatidylinositol biphosphate (PIP2) to triphosphate (PIP3). PIP3 activates phosphatidylinositol-dependent kinase (PDK1) which then phosphorylates protein kinase B (PKB), among others. PKB is then central in de-inhibiting: the translocation of GLUT4 vesicles; the activation of glycogen synthase (GS); and activation of ATP Citrate Lyase (fatty acid synthesis). It also activates mTORC1, promoting protein synthesis and cell growth and proliferation, and activates SIK2, inhibiting gluconeogenesis. (Weiss et al., 2000)

The IR is de-phosphorylated by protein tyrosine phosphatase 1B (PTP-1B) at the tyrosine residues of the beta subunit. This blocks IRS-1 binding and interrupts the signal cascade. When insulin concentration is low the auto-phosphorylation rate of IR drops, PTP-1B activity allows for a rapid modulation of glucose uptake. IR signalling is, in this way, dynamically regulated. Other phosphatases act on IR in a similar fashion, (Weiss et al., 2000)

1.2. On Vanadium

1.2.1. In nature and health

It was in the year of 1801 that the many colours of Vanadium (V) ion solutions led its first discoverer, Andrés Manuel del Río, to initially call it “Panchromium” (“of all colours”), later suggesting the name “Erythronium” (“red”) referencing one specific salt of V. This discovery was later retracted, and in 1831 a second description of the element was made by Nils Gabriel Sefström, naming it “Vanadium” in relation to the Norse goddess Vanadís (Freyja), known for her beauty. (Cintas, 2004) Vanadium has atomic number 23 and sits on the 4th period, Group 5 of the periodic table of elements. It is a transition metal with relative atomic weight of 50.94, and is noteworthy for its wide range of oxidation states, from +2 to +5. This allows it to behave as anion or cation, resulting in a large variety of compounds with greatly varying chemical properties. This chemical versatility is part of the reason why it has attained so much relevance in biology, ecology and health sciences, despite its relatively low abundance in the biosphere and the ongoing discussion on its essentiality in humans, as well as several other organisms. (Tracey et al., 2007)

The 22nd most abundant element in the Earth’s crust (0,019% among all discovered elements) and 5th most abundant transitional metal, it is ubiquitous in soil, water, air and life, largely in its tetravalent and pentavalent states; vanadium pentoxide (V_2O_5) being the most common and economically relevant compound. (Adriano, 1986; Imtiaz et al., 2015) It is reported by the European Commission as one of 27 raw materials of critical strategic importance due to its high demand and often difficult to replace role in industrial applications, versus its globally dependent supply chain with geopolitical concerns. (Petranikova et al., 2020) Worldwide, most Vanadium is obtained as a co-product of iron processing for steel production (71% of all production in 2019); it can be extracted directly via mining (18% in 2019); and it can be retrieved from secondary sources such as utility ash, petroleum residue or recycled spent catalysts (11 % in 2019). (Petranikova et al., 2020) Close to 80% of global supply is employed as an additive in steel alloys with broad use such as in the automotive, shipyard and aeronautical industries; it is also prevalent in the production of glass, catalysts, pigments, fertilizers, new-generation batteries, superconductive magnets; and, although to a much lesser extent, has been highly relevant in several scientific research areas, namely in biomedical and health sciences. (Imtiaz et al., 2015)

Owing to its plenty on the upper continental crust (100 ppm), and the relatively high solubility of its natural salts, vanadium is also abundant in seawater, averaging at 2 mg/m^3, much more so than iron. As such, it is understandable that throughout the evolution of biologic systems it came to be integrated in the metabolic processes of several organisms. (Pessoa et al., 2015) Numerous studies have concluded as to its essentiality or benefit to various bacteria, cyanobacteria, algae, fungi, lichen, plantae and animals. Enzymes such as bromoperoxidases in algae, haloperoxidases in several macro-algae, nitrogenases in nitrogen fixing bacteria, and chloroperoxidases in some fungi require Vanadium to correctly regulate their activity. (Rehder, 2020) In plants, studies show correlated increases between vanadium intake and metabolic activity, growth and yield in sugar

beet and maize, to name a few. However, it can also readily produce a toxic effect and decrease growth, such that its essentiality in plants is still unsettled. (Singh & Wort, 1969) Several animals have important metabolic roles for vanadium, which was reported in 1971 as an essential trace element in rats and chicks and in 1989 in goats. Symptoms of vanadium deficiency have been described elsewhere in birds, rodents, fish, and lower animals. Ascidians, in particular, and several other aquatic animals have specialized cells – Vanadocytes – for the accumulation of vanadium, which can reach much higher intracellular concentrations relative to their environment. (Michibata, 1996)

Under normal circumstances the average concentration of vanadium in the human body is 0.3 µM, and intake sources are drinking water, mostly, and food, with an average daily intake between 0.01 and 0.03 mg. (Pessoa et al., 2015) Most of this dietary vanadium is excreted in faeces. Its essentiality in human diet has been greatly debated but remains largely unproven. (Nielsen & Uthus, 1990) Proponents of this hypotheses point to its extensive physiological interaction, along with its essentiality in other mammals and higher animals and its ubiquity. It has been classified as essential by the American Dietetic Association. On the other hand, no specific physiological role has been found for V in humans such as exists in other organisms, no symptoms of its deficiency have been reported and, inversely, its acute and chronic toxicity has been extensively reported. (Ścibior et al., 2020)

1.2.2. Sources of Vanadium Exposure and Toxicity

Humans are continually exposed to vanadium from natural sources and also as a result of industrial activity. Natural dietary exposure is usually considered innocuous owing to the relatively low concentrations at which it occurs in food and water, as well as its low enteral absorption rate. While a daily oral intake below 10 $mg \cdot kg^{-1}$ exhibits no relevant physiological effects, food has an average content of 30 $\mu g \cdot kg^{-1}$, a negligible amount. (Treviño et al., 2019)

Natural phenomena like continental dust and seawater aerosolization or volcanism contribute some vanadium to the atmosphere, but this is generally residual and rarely hazardous. (Tripathi et al., 2019) In areas of low or no pollution vanadium oxides average at 0,8 to 1.2 $ng \cdot m^{-3}$ of air. (Rehder, 2015) Industrial activity such as mining, metallurgy, chemical and catalyst production, petroleum refinement, fossil fuel combustion and waste management, releases vanadium, locally increasing its concentration in air. (Dill, 2003; Rehder, 2015) Occupational exposure by workers in these industries is of great concern since they are at risk of acute V intoxication through inhalation, and are at greater risk of suffering from chronic effects as continuous low dose exposure may be asymptomatic for years before noticeable problems arise. Maintenance workers for fossil fuel thermal power boilers, high-pressure combustion engines and vanadium based catalytic converters are among the most susceptible to highly toxic species. (Rehder, 2015)

The toxicity of V has been known for almost as long as it has. It is one of the main concerns when developing applications in medical sciences, either in alloys or for its pharmacological interest. Its toxicity is mainly dependent on oxidation state, since the bioavailability and accumulation of different ions differs so greatly. The +5 oxidation state, such as in vanadate, is the most toxic. Vanadium pentoxide, a common environmental and occupational hazard, is a common source of pulmonary intoxication since it readily dissolves and diffuses in the alveolar surface, then entering the bloodstream. (Pessoa et al., 2015) As mentioned before, normal dietary intake has a very low probability of reaching effective levels, however, intoxication may happen through contamination of food and water, or deliberate consumption (e.g. in clinical trials with V compounds).

Exposure via enteral routes may lead to gastrointestinal disorders like diarrhoea, nausea, vomiting, abdominal pain, appetite and weight loss and green tongue. Inhalation may cause pharyngitis, bronchitis, dyspnoea, asthma, pneumonia, migraine, conjunctivitis and depression. (Ścibior et al., 2020)

Vanadium increases oxidative stress in the body by interacting with other oxidants in tissues and cells. This in turn can disrupt cell membranes, denature essential proteins, increase lipid peroxidation, and cause DNA degradation. It can increase free transition metals in the body by displacing them in transporters. Because it accumulates in many organ tissues, increasing oxidative stress, it has hepatotoxic, nephrotoxic, cardiotoxic, and neutoroxic action. Chronic pulmonary exposure produces inflammation and pulmonary fibrosis. Vanadium is a category 2 carcinogen, and long-term exposure increases oxidative stress and mutagenic effects on cells. Pre-natal exposure effects have also been reported. (Rehder, 2020)

Its interference with kinase and phosphatase activity affects many metabolic processes. Where vanadate can substitute phosphate in coordination structures, it does so with greater affinity. Phosphate interactions with biomolecules are usually reversible, while vanadate often binds these irreversibly. The consequences of this are numerous and not fully understood, as so are not many of the mechanisms of toxicity of V.

1.2.3. Current and Proposed Medical uses

There is an actively growing body of work on potential uses for different compounds and metallic alloys in medical and biomedical applications, although their current clinical use is essentially restricted to prosthetic alloys and implant coatings. Two Ti-Al-V alloys are currently in use in orthopaedic and dental prosthetics and one Co-Fe-V alloy is used in heart implantable devices. (Davis, 2003) Even though vanadium in these alloys contributes to achieve specific mechanical and chemical properties, surface coatings are applied to both block metal leaching out of the prosthesis and improve osteo and tissue-integration, owing to their toxic potential.

Polymeric implant coatings, such as Poly-LactoGlycolic acid (PLGA) complexed with vanadium, are also in current use. (Davis, 2003)

A possible anti-viral effect in HIV-1, HIV-2, influenza and SARS infection has been proposed. However, this has been mostly based off of in vitro studies where complexes show inhibition of HIV reverse transcriptase, decreasing viral replication. (Rehder, 2013) Anti-parasitic action for Trypanosoma, Leishmania and Entamoeba has also been observed in vitro, possibly caused by DNA intercalation, phosphatase competitive inhibition or macrophage activation. Vanadium complexes have also been studied for anti-fungal activity against Aspergillus, Candida and Trichophyton, and gram-positive and gram-negative anti-bacterial activity. (Rehder, 2013)Various studies have remarked on anti-neoplastic actions, suggesting that reactive oxygen species (ROS) generation by vanadium-ferritin interaction may increase cell apoptosis in tumours. Neoplastic transformation suppression was observed in vivo, and decreased metastasis in vitro with several cell lines. Kowalski et al. reported that a 2-methylnitrilotriacetate ligand coordinated with oxovanadium complexes has potential for use in pancreatic cancer preclinical research. Many other compounds have been similarly assessed. (Pessoa et al., 2015)Cardiovascular effects, for example in prevention of hypertension and myocardial hypertrophy in animal models, have been proposed for vanadyl sulfate ($VOSO_4$) or bis(maltolato)oxovanadium (BMOV). Studies on appetite regulation by BMOV administration in rats, revealed that its effect on leptin signalling led to reduction in food intake and body weight. Vanadium compounds have also displayed inhibition of cholesterol synthesis in vitro, pointing to a possible anti-hypercholesterolaemic use. (Bhuiyan & Fukunaga, 2009)

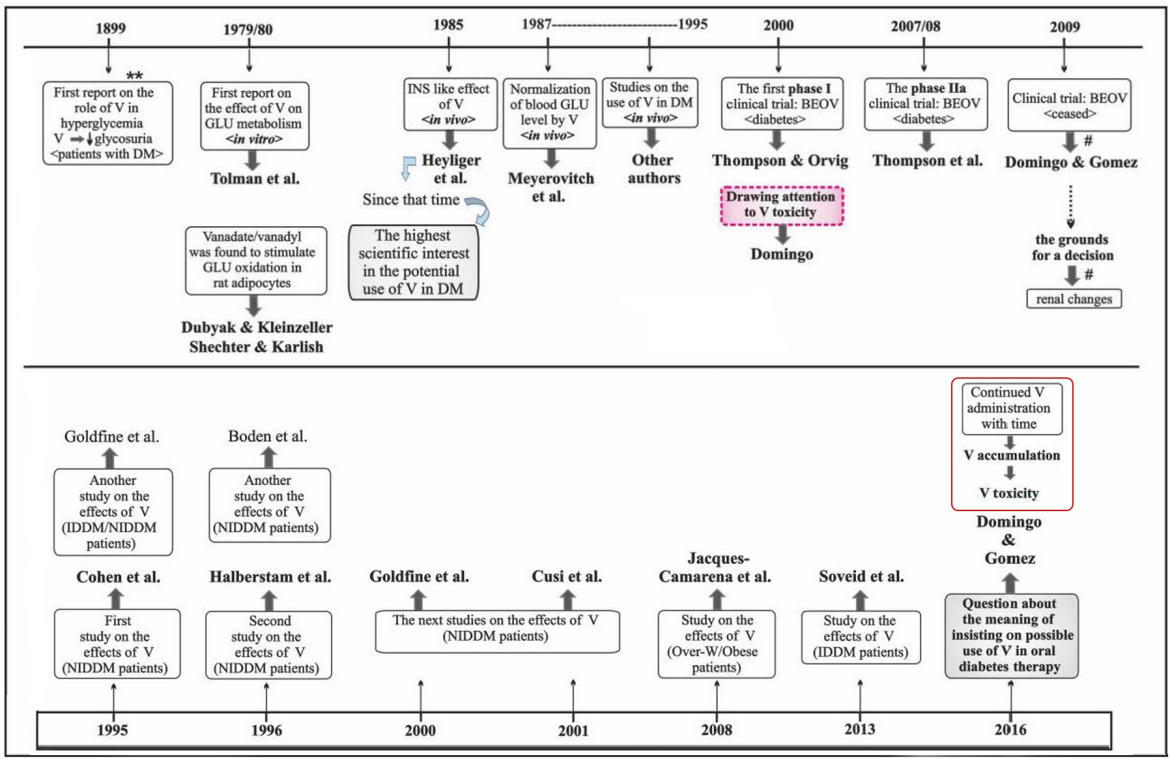


Figure 1: Overview of past research on the antidiabetic effects of Vanadium compounds. Adapted from (Ścibior et al., 2020)

As is apparent, Vanadium has been suggested and studied for a numerous and varied spectrum of applications in health sciences, none more so than for its possible antidiabetic effects, which have made up a substantial part of published research.

1.2.4. Antidiabetic effect in literature

The potential of Vanadium as an antidiabetic, whose research history has been summarized in figure 1, was first reported by Lyonnet et al. in 1899 after experimenting over some months, initially on the research team and later on 60 patients, by administering 4 to 5 mg metavanadate over 24 hours, three times a week, orally before meals. Lyonnet reported a small decrease in glycaemia and glycosuria in two of the three diabetic patients, without negative effects. (Thompson & Orvig, 2006) Although of dubious methodology by today's standards, it spurred interest in the field. After the discovery and purification of insulin in 1921 research in this field fell somewhat silent as a straightforward therapeutic solution for diabetes had been achieved. In 1979 new research (Tolman et al., 1979) finally showed a clear association between vanadium and glucose metabolism in vitro and in 1980 other works showed a stimulation of glucose oxidation by vanadyl and vanadate compounds in rat adipocytes. (Dubyak & Kleinzeller, 1980)

The first description of antidiabetic effects in vivo was made by Heyliger (Heyliger et al., 1985), using diabetic rats. This renewed interest on the subject, and the body of work steadily started to mount. In vivo research using vanadyl sulfate, sodium orthovanadate (Na_3VO_4), sodium metavanadate ($NaVO_3$) and other salts occurred over the following years. Meyerovitch reported glycaemia normalizing effects of orally administered vanadium in streptozotocin-treated rats (STZ rats) in. (Meyerovitch et al., 1987)

Only in 1995 was the first methodologically modern study in humans with diabetes performed. Cohen et al. tested patients with type II diabetes to assess whether insulin sensitivity was altered by vanadyl sulfate *per os*. (Domingo & Gómez, 2016) The six subjects' insulin sensitivity improved for up to 2 weeks after the last administration, but five of them experienced mild gastrointestinal side effects during the first week of treatment. Shortly after, also in 1995, Goldfine et al. reported on the effects of sodium metavanadate in five type I and five type II patients, on a two week oral regimen. The observed increase in MAPK and S6K activity in both groups mirrored that of the insulin control groups. (Domingo & Gómez, 2016) Gastrointestinal side effects were again reported in several subjects. Halberstam et al. in 1996 showed that between two groups of subjects with obesity, one type II diabetics, the other non-diabetics, that only the diabetic group's insulin sensitivity was improved by a vanadyl sulfate oral regimen, also reporting mild gastrointestinal side effects. That same year, Boden et al. tested type II diabetic subjects, first on a 4-week oral vanadyl sulfate regimen followed by a 4-week equivalent placebo regimen. Fasting glycaemia and hepatic gluconeogenesis decreased throughout both periods, gastrointestinal side effects were noted. (Domingo & Gómez, 2016)

These various studies pointed towards a trend: that there might actually be significant antidiabetic effects to vanadium compounds. But the therapeutic window of inorganic vanadium salts, particularly vanadyl sulfate, is too small for effective, safe clinical use.

1.2.5. Proposed Mechanisms of Action

Of the currently proposed mechanisms to explain the antidiabetic action of vanadium compounds, the most agreed upon relates to the inhibition of PTP-1B. As previously mentioned, when insulin concentration is low PTP-1B de-phosphorylates the tyrosine residues at the beta subunits of the IR, thus blocking IRS-1 from binding to the IR, interrupting the signalling cascade, PTP-1B is deactivated by phosphorylation of a cysteinyl residue at its active centre. (Rehder, 2020) The structural similarity of vanadate, one of the intracellularly bioavailable species of V, to phosphate allows it to bind to this residue, however, this coordination is more stable than normal phosphorylation, irreversibly deactivating PTP-1B. The IR phosphorylation ratio therefore increases, and the signalling cascade increases in sensitivity. For this reason, some authors argue that vanadium is not so much an insulin-mimetic, but more a signal modulator or insulin enhancer, for without the simultaneous activation of the IR by insulin the signal transduction would be insufficient. Another proposed mechanism is that ROS created in vanadium metabolism also acts upon PTP-1B, inhibiting it. Vanadium has also been shown to deactivate various other phosphatases by coordination with their active centres, e.g. SHP-1, SHP-2 and the PTP associated with insulin-like growth factor receptor (IGF-IR), which may potentiate its antidiabetic effect but also causes concerns as to its specificity of action. Moreover, the interaction of V with several membrane and cytosolic proteins may also be a factor in both its beneficial effects and potential for adverse ones. (Rehder, 2020)

1.2.6. Organic Vanadium complexes

Research with greater focus on vanadium bound to organic ligands currently has the most momentum, with the goal of providing a way to administer it with a safer and more tolerable pharmacokinetic profile and a lower therapeutic dose so as to circumvent toxicity concerns. Mcneill (McNeill et al., 1992) first reported on the potential of BMOV as an insulin-mimetic, owing to it having up to three times more pronounced effects in decreasing glycaemia compared to vanadyl sulfate; a more tolerable therapeutic profile; overall more consistent effects in various animal models; and the possibility of delivery through a simple aqueous solution. (Thompson et al., 1998) By 2000, several ligand complexes had already been synthesized in the search for a more insulin-mimetic and yet safe compound. That year, bis(ethylmaltolato)oxovanadium (BEOV) entered a phase I clinical trial as an antidiabetic prodrug, during which no adverse effects or organic anomalies were reported. (Thompson & Orvig, 2006) In 2007 it was further tested in a phase IIa clinical trial on seven type II diabetic patients. The clinical development program for the

AKP-020 (BEOV's trial designation) candidate, and others involving derivatives of BMOV, were eventually discontinued in 2009 citing renal alterations observed during a preclinical safety program. (Domingo & Gómez, 2016)

A systematic review from 2008 by Smith et al. aimed at evaluating research on type II diabetes glycaemic control by oral vanadium compounds. Specifically, it searched relevant databases for studies with randomized, placebo controlled trials using oral vanadyl sulfate at 30-150 mg per day for at least 2 months, with a minimum sample size of 10 patients per trial group. Studies that failed any single one of these parameters were also included. From a total of 151 retrieved papers, 5 met the criteria for further analysis and of these none actually met the inclusion criteria for the review. The authors concluded that there was, therefore, insufficient evidence that oral vanadium administration has any beneficial effect in glycaemic control in patients with type II diabetes. (Smith et al., 2008)

As to its effectiveness and safety in type I diabetes patients, Soveid et. al. conducted a study of 14 patients dosed with oral vanadyl sulfate, initially for 2 to 5 weeks with 80-120 mg per day, followed by 30 months with 225-300 mg per day, during which average insulin requirements decreased 30%, fasting glycaemia decreased substantially, and no adverse effects were recorded other than diarrhoea at start of treatment. (Soveid et al., 2013)

In a review paper in 2016, Domingo & Gómez classify this field of research as "scientific curiosity" without practical application for treatment based on the issue of long-term toxicity of Vanadium. It is argued that most research on human subjects has been focused on short-term results and adverse effects have been measured accordingly, while diabetes mellitus is currently an incurable condition with lifelong therapeutic management. Hence, any therapeutic applications of V compounds should require continuous long-term administration, which ultimately would result in bioaccumulation and chronic exposure to a proven highly toxic metal. (Domingo & Gómez, 2016) Furthermore, diabetes is a metabolic disease with systemic physiological effects and a great number of associated complications, and this has yet to be taken into account since most research has focused solely in the insulin-mimetic action of V.(Domingo & Gómez, 2016; Ścibior et al., 2020)

Human trials have used much lower doses than animal studies, and correspondingly have less pronounced effects than those reported in animals. Additionally, any intent at producing effective and safe therapeutic drugs from these inorganic salts that could be administered over great spans of time, such as is needed in diabetes management, is immediately met with the problems of their biodistribution and accumulation in most critical organs of the body and their ample interaction with organic molecules. (Goldwaser et al., 2000)

Using organic ligands to chelate V into compounds with better bioavailability and antidiabetic effect at low doses, stable coordination structures with less release of free V ions, and more targeted physiological interactions, has become the main focus of the last 20 years of research. Many different complexes with various coordination structures and functional groups have been

synthesized, characterized and tested in vivo and in vitro. A brief overview on some of them will be given in this section.

Almost all ligands have been synthesized for coordination with VO2+ since this ion is less toxic than vanadate, has more affinity for coordination with blood and cell membrane transporters, has a lower residence time in the body and higher renal clearance, while at the same time binding more stably to organic ligands and showing more consistent antidiabetic effects. As discussed earlier, VO2+ is overall less enterally absorbed, so that oral delivery is ineffective, this can be surpassed through chelation with more bioavailable compounds. (Makinen & Salehitazangi, 2014)

The aforementioned BMOV and its ethyl analogue BEOV were some of the first complexes developed for this purpose and in STZ rats showed better results in both absorption and glycaemic lowering effect compared to vanadyl sulfate. They both eventually entered into clinical trial programs (previously described). Because of this early arrival and success, as well as the amount of research, they have become a common control in other studies. (Treviño et al., 2019) This class of complexes have the vanadyl group bond to two bidentate ligands with a 3-hydroxy-4-pyrone core coordinated with different functional groups, methyl in BMOV, ethyl in BEOV for example. Others in this class include VO(isopropylmaltolato)$_2$, VO(n-butylmaltolato)$_2$, VO(kojato)$_2$ and VO(allixinato)$_2$. Although structurally similar, the functional groups on the pyrone ring lead to differences in solubility, stability, bioavailability and glycaemic effect. Considerable attention has been given to these complexes, and several of them show results in line with those of BMOV. VO(isopropylmaltol)$_2$ was reported to produce equivalent glycaemia decreases at lower V levels, while VO(allixinato)$_2$ has an average blood residence time in rats 2 to 1.8 times longer than BMOV or BEOV. (Makinen & Salehitazangi, 2014)

Another proposed class uses bidentate ligands with a 3-hydroxi-4-pyridinone (pp) core structure. VO(dimethyl-pp)$_2$, VO(methyl-pp)$_2$, VO(ethyl-pp)$_2$, VO(dimethyl-pp)$_2$ and VO(ethyl-methyl-pp)$_2$ have all been assessed for insulin-mimetic effects. In vitro studies showed that VO(methyl-pp)2 has a higher antidiabetic effect than vanadyl sulfate, as measured by inhibition of free fatty acid (FFA) release. VO(dimethyl-pp)$_2$ increases in vitro adipocyte glucose uptake more than BMOV, and chronic treatment of GK rats decreased hyperglycaemia, improved tolerance to glucose and did not cause hypoglycaemia in normal wistar rats. (Domingues et al., 2014; Rangel et al., 2009)

Bidentate ligand complexes of acetylacetonate (VO(acac)$_2$) 3-alkyl-acetylacetonate analogs have also obtained interesting results. STZ-diabetic rats acutely treated with oral and injected VO(acac)$_2$ have shown an up to 5 day sustained decrease in glycaemic levels. This long acting effect is attributed to the compound's high stability and its interaction with serum albumin, which increase its blood residence time. (Makinen & Salehitazangi, 2014)

Picolinate (pic) and derivatives such as dipicolinate (dipic), several methylpicolinates (Xmpic), Iodopicolinate (5Ipic) and chloropicolinate (4Clpic), bidentate ligands, are also reported to have

various degrees of effectiveness. $VO(pic)_2$ showed insulin-mimetic effects on in vitro adipocytes and is capable of normalizing STZ rat glycaemia through daily oral or ip injection administration. (Sakurai, 2002)

Recently, Szklarzewicz et al. has reported that tridentate ONO Schiff base ligands produced from hydroxi-salicylaldehyde, dichloro-salicylaldehyde, dibromo-methoxy-salicylaldehyde and various hydrazides, together with a phenantroline co-ligand can form very stable complexes with an inhibitory effect on PTP-1B, SHP-1, SHP-2 and LAR. This points to an antidiabetic effect, however, this has not been tested in in vitro cell models nor in vivo. (Szklarzewicz et al., 2021)

Overall, research on these, and other, oxovanadium complexes has not been conclusive as to its application in human diabetes. Even so, several insights into the pharmacokinetics and insulin-mimetic mechanism of action of V have been gathered. It also has become apparent that, aside from insulin signal activation or enhancement, compound stability and interaction with transport proteins are important factors in their action. Particularly, in the interest of developing orally administrable insulin substitutes, compounds must be stable at least at intestinal pH, presuming drug encapsulation is used to avoid exposure to the gastric environment. The de-coordination of ligands due to interactions with food and body proteins may change the activity of the complex or even release V species in free form. (Domingo & Gómez, 2016)

1.2.7. Oxovanadium Pyridinone Complexes

Previous work focusing in the insulin mimetic effects of vanadyl complexes, such as VO(mpp)2 [bis(3-hydroxy-2-methyl-4(1H)-pyridinone)VO], VO(dmpp)2 [bis(1,2-dimethyl-3-hydroxy-4(1H)-pyridinone)VO] have suggested that these compounds may have important roles in this area, especially because they seem to be less toxic than other vanadium complexes such as $VOSO_4$ (Domingues et al., 2014; Passadouro et al., 2010; Rangel et al., 2001).

Domingues, *et al.* have demonstrated the in vitro and in vivo insulin mimetic effects of $VO(dmpp)_2$ in a T2D animal model. They have found that $VO(dmpp)_2$ improves glucose uptake in primary adipocytes of Wistar and the Goto-Kakizaki (GK) rats. This was also seen in primary adipocytes of Wistar rats, by Passadouro *et al*.. Furthermore, in a chronic in vivo treatment, $VO(dmpp)_2$ was able to decrease hyperglycaemia, and improve glucose tolerance significantly. It is shown that these effects are due to the direct effect of $VO(dmpp)_2$ on proteins of the insulin signalling pathway, specifically increasing IRS2 expression and AKT phosphorylation and inhibiting PTP1β expression (Domingues et al., 2014).

A study by Rangel, *et al*, focusing on Vanadyl complexes, including $VO(mpp)_2$, $VO(dmpp)_2$ among others, and using the vanadyl sulfate as a control, reported that all the complexes have an inhibitory effect on FFA release. Two pyridinone complexes, $VO(mpp)_2$ and $VO(empp)_2$, have a significantly better insulin-mimetic activity than that of vanadyl sulfate (Rangel et al., 2001).

2. Main Goal

In light of current knowledge in the field of Vanadium antidiabetic compound research, the present work aims to further understanding of how oxovanadium complexes impact glucose metabolism. Specifically, how much $VO(dmpp)_2$ and $VO(mpp)_2$, two Vanadium bidentate pyridinone ligands, influence overall adipose tissue metabolism and insulin signalling activation, upon stimulation with differing concentrations of these compounds, as compared to equivalent concentrations of $VOSO_4$, in the presence or absence of insulin.

3. Materials and Methods

3.1. Experimental Design

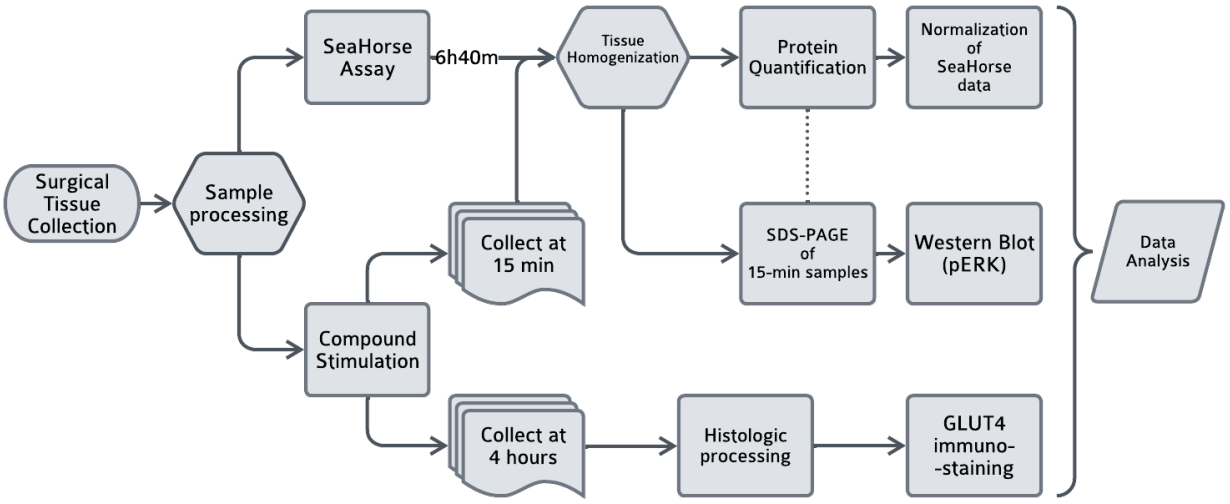


Figure 2: Experimental design algorithm.

In order to tackle this question an experimental plan was designed, as per figure 2, that could provide insight into both the compounds' overall metabolic and mechanistic effects. To this effect, human visceral adipose tissue was to be collected, processed and set to be studied in response to the compounds in either a bioenergetics assay (SeaHorse assay) to measure oxygen consumption metabolism; or to determine how the insulin signalling pathway could be affected, by evaluating the change in phosphorylation of ERK – via western blot – and translocation of GLUT4 – through immunostaining of tissue slides.

For each of the tested compounds – $VOSO_4$, $VO(mpp)_2$, and $VO(dmpp)_2$ (henceforth labelled VOSO, MPP and DMPP, respectively) – three concentrations were decided upon, as informed by the relevant literature: 50 µM; 100 µM; and 500 µM. All conditions were supplemented with insulin to access the differing modulation of insulin signalling. Media with insulin was used as the control condition.

3.2. Synthesis of Vanadium compounds and preparation of solutions

Vanadium complexes were synthesized, characterized and provided by the Department of Chemistry at ICBAS. Briefly, stoichiometric quantities of vanadyl sulfate and each pyridinone ligand were added in water, pH adjusted to 5, and refluxed for approximately 1 hour. The bluish precipitates that formed were then filtered before cooling, washed in methanol-water, and lastly dried over phosphorus pentoxide.

The day previous to cell exposure, the required mass of each powdered compound was weighed and left to UV-sterilize inside the tissue culture hood. For each of the compounds, solutions were prepared the day of use, by first preparing a 5×10^{-4} M solution, with 1% dimethyl sulfoxide (DMSO) to improve solubility. The DMSO was added to each compound and briefly vortexed at 1500 rpm. Afterwards, media was added up to the required volume. The solutions were placed in an orbital shaker at 1200 rpm, 37°C for 10 min, to ensure complete dissolution. From this, dilutions at 10^{-4} M and 5×10^{-5} M for each compound were prepared from the primary solutions by adding media with 1% DMSO. Lastly, insulin was added to each solution to a concentration of 1 µM.

3.3. Adipose tissue collection

Subjects undergoing elective laparoscopic bariatric surgery (n=5) for the primary treatment of obesity, and related co-morbid conditions, at *Centro Hospitalar entre Douro e Vouga* (CHEDV) were invited to take part in the study. Patients who accepted and did not meet any of the exclusion criteria (ongoing pregnancy, active acute infectious conditions or prior history of neoplastic diseases) were included and provided written informed consent for the surgeon to perform a small visceral adipose tissue (VAT) biopsy. All experiments in this study were performed after approval by the Ethics Committee of CHEDV and in accordance with the relevant guidelines and regulations.

Following explant collection, vascular tissue and burnt areas were removed and VAT biopsies were transferred into Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12; Sigma-Aldrich, St Louis, MO) supplemented with 10% Foetal Bovine Serum (Sigma-Aldrich, St Louis, MO) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St Louis, MO). Samples were then transported to the laboratory for immediate processing.

After processing and assay completion, the tissue samples were collected into a 1.5 mL tube and stored at -20°C for further analysis.

3.4. Seahorse assay

The day prior to the experiment, the Seahorse sensor cartridge was hydrated with a commercial calibration solution (XF Calibrant pH 7.4, Agilent Technologies, Santa Clara, CA, USA), at 37°C in an incubator, absent CO_2. Upon arrival of samples, the VAT fragments (approximately 5 mg each) were deposited and attached by capture screens at the bottom of the cartridge's wells and incubated in a commercial medium (Seahorse XF DMEM Medium, pH 7.4, 103575 – 100, Agilent Technologies) for at least 45 minutes at 37°C, absent CO_2.

Meanwhile, the electron transport chain modulator solutions were prepared and transferred to the cartridge. Medium with DMSO, final concentration of 0.2% (D2650 – 5X10ML, Sigma Aldrich) was used as control. Ports A, B and C of the cartridge were filled thusly: A – oligomycin solution (final concentration 12.5 µg/mL, O4876-25MG, Sigma Aldrich), the ATP-synthase inhibitor; B – FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, final concentration 8 µM, C2920-50MG, Sigma Aldrich), the mitochondrial oxidative phosphorylation uncoupler; C – a combination of Rotenone (final concentration of 2 µM, R8875-5G, Sigma Aldrich) and Antimycin A (final concentration of 4 µM, A8674-100MG, Sigma Aldrich), mitochondrial complexes 1 and 3 inhibitors, respectively. The cartridge was then mounted on the Seahorse analyser (Seahorse® XFe24 Analyzer, Agilent Technologies) for cartridge validation.

VAT samples were incubated in seahorse assay medium supplemented with 0.01 µM glucose (103577 – 100, Agilent Technologies) and 2 µM glutamine (103579 – 100 Agilent Technologies) and prepared according to the aforementioned experimental conditions. Conditions were assayed in duplicates. The plate was mounted in the Seahorse analyser and, after a stabilization period of 45 minutes, the electron transport chain modulators were released into the samples, sequentially, as per the programmed timings.

The oxygen consumption rate (OCR) of the VAT samples was registered, real-time, for a total of approximately 6 hours and 40 minutes. Oligomycin was added at the 45th minute mark; FCCP at 105 minutes; and rotenone/antimycin A at 224 minutes. After assay conclusion, results were exported to the Seahorse Wave 2.6.3 software, and data for each condition was later normalized by the amount of protein in each sample.

All concentrations and timepoints used in this study had been previously optimized by the research group.

3.5. Tissue stimulation for GLUT4 translocation/Phospho-ERK western blot assays

On the day of collection, tissue was divided into fragments of roughly 50 mg. One such fragment was directly collected in formaldehyde (Pancreac, Barcelona, Spain), as 0-hour control. Other VAT fragments were placed in 48-well plates, for stimulation in the previously described experimental conditions. Two sets of stimuli were performed: one collected at t+15 minutes and frozen at -20°C for protein extraction and western blot analysis; and another collected at t+4 hours and immersed in formaldehyde for further histological processing.

3.6. Tissue Homogenization and Protein Extraction

Tissue samples were homogenized following a modified Removal of Excess Lipids (RELi) protocol (Diaz Marin et al., 2019), in order to better assure protein extraction with low lipidic contamination for quantification. The previously frozen samples were transferred to 2 mL capped tubes with Zirconia/Silica microbeads (Biospec Products, UK). Seahorse assay samples had 300 µL RIPA buffer (Sigma-Aldrich, St Louis, MO, USA) with protease inhibitor (ref: 4693124001; Roche, Basel, Switzerland) and phosphatase inhibitor (ref: 4906845001; Roche, Basel, Switzerland) added and proceeded to tissue lysis. Samples stimulated for Phospho-ERK western blot assays, due to their larger size, had 350 µL of this same buffer added and were left overnight on a tube rotator at 4°C before lysis.

Samples were kept on ice both before and at least 1 minute in between lysis cycles. Lysis was accomplished using a FastPrep®-24 (MP Biomedicals, USA), on cycles of 30 seconds at 6.0 m/s. Seahorse samples required 4 cycles for complete lysis while tissues for western blot required 5. They were then incubated on ice for 1 hour. After incubation samples were centrifuged at 20000 g, 4°C for 15 minutes and the liquid fraction pipetted out to a 1,5 mL tube with care to not transfer the lipidic top layer. Centrifugation was repeated two more times, each time the top lipid layer discarded and the remainder transferred to a new 1,5 mL tube.

3.7. Protein Quantification

The Pierce™ BCA Protein Assay Kit (ref: 23225; ThermoFisher Scientific, Waltham, MA, USA) was used for protein quantification, with minor modifications to protocol. The microplate procedure was used with 96-well plates. In Seahorse samples, 25 µL of standards and samples were used for quantification, while in tissue for western blot only 10 µL, in order to preserve sufficient sample volume for electrophoresis. Despite reducing the assay's working range in some samples, all protein concentrations fell well within range.

For each well, 200 µL of the kit's working reagent were added, and the plate incubated at 60°C for 30 minutes instead of the recommended 37°C. This allowed for more reliable results in the present work. After cooling, plates were loaded into a microplate reader (Thermo Scientific™ Multiskan™ FC Microplate Photometer, ThermoFisher Scientific), briefly shaken, and absorbance measured at 570 nm. The standard curve was fitted using the microplate reader's quadratic regression algorithm.

3.8. Translocation of GLUT4

The t+4 hour incubated fragments, immersed in 4% buffered formaldehyde, were preserved for 72 hours at room temperature, and subsequently processed for paraffin embedding and optical microscopy.

Briefly, 3 µm formalin-fixed paraffin embedded tissue sections were mounted on adhesive microscope slides (StarFrost, Knittel Glass, Germany), deparaffinized, rehydrated in graded alcohols, and microwave heated at 900 W in 10 mM citrate buffer (pH 6.0) for 15 min, for antigen retrieval. Auto-fluorescence inhibition was performed by incubation with Sudan black B 0.5% in 70% alcohol for 30 min. Slices were incubated overnight with primary anti-Glucose transporter GLUT 4 antibody (ab33780, Abcam, Cambridge, UK) (1:100) at 4 °C. Slides were then incubated for 2 h with a fluorescent secondary antibody anti-rabbit (1:1000, #555, Cell Signalling Technology, Danvers, MA, USA). These were then mounted and counter-stained with DAPI hard set (ref. H1500, Vector Laboratories, Burlingame, CA, United States).

Slides were exposed and imaged using a Nikon® NIS-Elements 5.41.01 imaging software.

3.9. Phospho-ERK western blot

Samples collected at t+15 min stimulation were thawed, homogenized and protein extracted and quantified as previously described. Afterwards, a total 20 µg of protein was heated at 95°C for 5 minutes, fractioned on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Nitrocellulose membranes. Membranes were blocked in Tris-buffered saline solution with 0.05% Tween 20 containing 5% BSA (Sigma-Aldrich, St Louis, MO), and incubated overnight at 4°C with phospho-ERK (1:2000, 4370S; Cell Signalling Technology, Danvers, MA), or total ERK (4696S; Cell Signalling Technology, Waltham, MA, USA), separately. Immune-reactive proteins were detected separately using an anti-rabbit secondary antibody (1:5000, ab6721; Abcam, Cambridge, UK) for phospho-ERK detection and an anti-mouse secondary antibody (1:2000, ab97040; Abcam, Cambridge, UK) for total ERK detection. Membranes were reacted with ECL detection (ThermoScientific, USA) system and read with the Chemidoc MP Imaging System (Bio-Rad, Hercules, CA). Densities for each band were obtained with Image Lab Software 5.1 from Bio-Rad (Hercules, CA, USA). The band background adjusted intensity was divided by the corresponding total protein intensity quantified.

3.10. Statistical Analysis

Statistical analysis was performed using GraphPad® Prism software (USA), version 9.4.1 for Windows. Quantitative variables are expressed as mean ± standard error of the mean (SEM). Groups were compared using the Friedman test., with $p<0.05$ considered statistically significant.

4. Results

4.1. Patient Anthropometric Data

We invited a group of 5 obese patients undergoing elective bariatric surgery for visceral adipose tissue collection. Table 1 presents anthropometric data for the included patients. Of the selected cohort, none had a diagnostic of type II diabetes mellitus.

Table 1: Anthropometric and metabolic parameters of the participating patients.

Sex (F/M)	2/3
Age (years)	49,7 ± 2,51
Weight (kg)	114,3 ± 4,73
BMI (kg/m^2)	39,4 ± 2,23
FPG (mg/dL)	98,5 ± 4,67
HbA1c (%)	5,6 ± 0,17

4.2. Seahorse Assay

Upon completion of the Seahorse assays, the Wave analyser software outputs graphed data such as is presented in figure 3. These allow for a quick overview of the tissue response to the various phases of the assay. This graphed data was consistent throughout the samples.

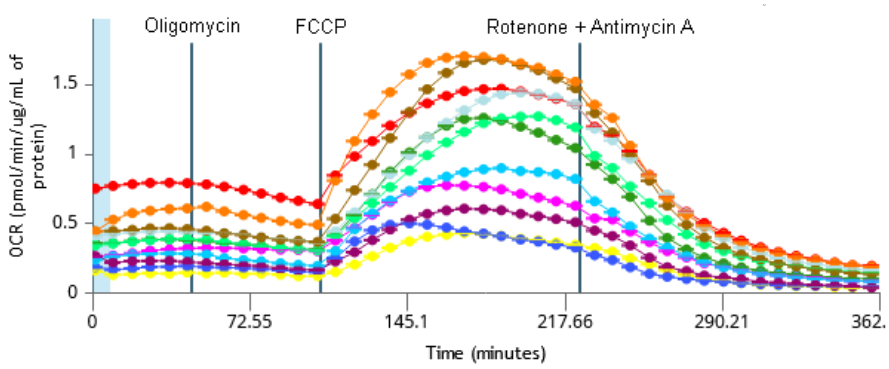


Figure 3: SeaHorse Metabolic Profile OCR curve for one of the samples.

The raw oxygen consumption rate data, was processed for the relevant parameters of cellular oxygen consumption, presented in table 2.

Table 2: Seahorse assay calculated OCR parameters, standardized by the INS condition.

	Total Base OCR		Non-Mito OCR		Mito OCR		MAX Resp		Spare Cap		ATP-coupled		Proton Leak	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM
INS	0.124	0.055	1	0.2785	1	0.5585	1	0.4881	1	0.4782	1	0.1856	1	0.7693
VOSO 1	0.326	0.102	3.184	1.05	2.412	0.7652	2.699	0.9424	2.796	1.022	1.932	0.5395	2.666	0.9127
VOSO 2	0.452	0.148	3.066	1.1	3.852	1.233	2.729	0.9009	2.347	0.9075	2.68	0.8068	4.472	1.478
VOSO 3	0.317	0.033	2.531	0.1584	2.56	0.3047	1.644	0.3542	1.332	0.3858	0.7695	0.3969	3.508	0.2806
MPP 1	0.241	0.021	2.227	0.1084	1.832	0.1985	2.015	0.1537	2.078	0.1457	1.402	0.5502	2.06	0.1606
MPP 2	0.283	0.081	2.911	0.8586	2.038	0.5831	2.326	0.7278	2.424	0.7854	1.347	0.5174	2.404	0.6528
MPP 3	0.251	0.064	3	0.9259	1.652	0.4046	1.84	0.6079	1.903	0.6899	1.355	0.5007	1.809	0.3775
DMPP 1	0.245	0.106	2.925	1.455	1.605	0.7398	2.069	1.028	2.227	1.142	1.686	0.6239	1.562	0.832
DMPP 2	0.198	0.04	1.994	0.9997	1.436	0.3177	1.466	0.3017	1.476	0.371	1.425	0.2929	1.441	0.4262
DMPP 3	0.317	0.108	3.019	0.9675	2.369	0.8294	2.361	0.7978	2.359	0.8023	1.824	0.7069	2.657	0.8965

Values are presented as averages and standard error means. For all parameters except for Total Base OCR, conditions were normalized by the average value of the INS condition. Total Base OCR was mainly calculated to assess viability and base metabolic activity. Samples in which this parameter was either negative or approximately 0 $pmol \cdot min^{-1}$ were excluded from calculations since their lack of metabolic activity did not afford a viable comparison to their respective replicas.

These values are graphed in figures 4 and 5. As can be observed, the Non-mitochondrial OCR increased for all conditions relative to the control, but no apparent relation to the concentration of the vanadium compound exists.

Mitochondrial OCR was elevated in comparison to control in all VOSO concentrations and DMPP 500 µM. There exists no clear relation to compound concentration.

All conditions saw an increase in MAX Respiration values, particularly in the case of the two lower VOSO concentrations. Once more, concentration did not have any discernible effect.

The spare capacity also did not see relevant increases in the vanadium conditions, except for a more marked increase in the VOSO 50 µM condition, again without proportionality to concentration.

ATP-coupled respiration was more prominent in the lower VOSO concentrations, especially VOSO 100 µM. While Proton leak remained somewhat stable between conditions, except for a prominent increase at the VOSO 100 µM.

However, despite these observations in the differing behaviour or lack thereof of the oxygen consumption profile of these conditions, no statistical significance was found in-between them.

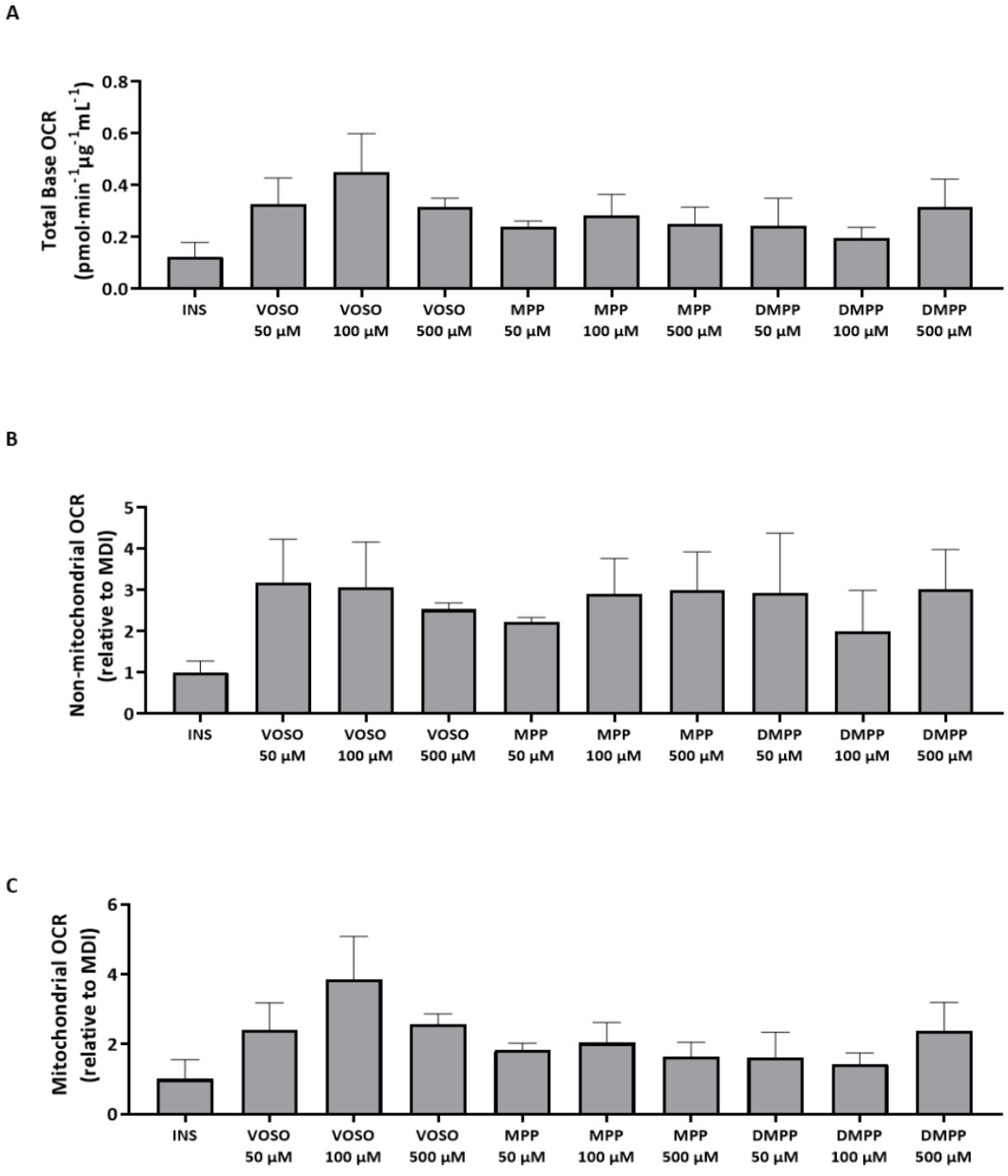


Figure 4: SeaHorse graphed parameter data. A: Total Base OCR; B: Non-mitochondrial OCR; C: Mitochondrial OCR

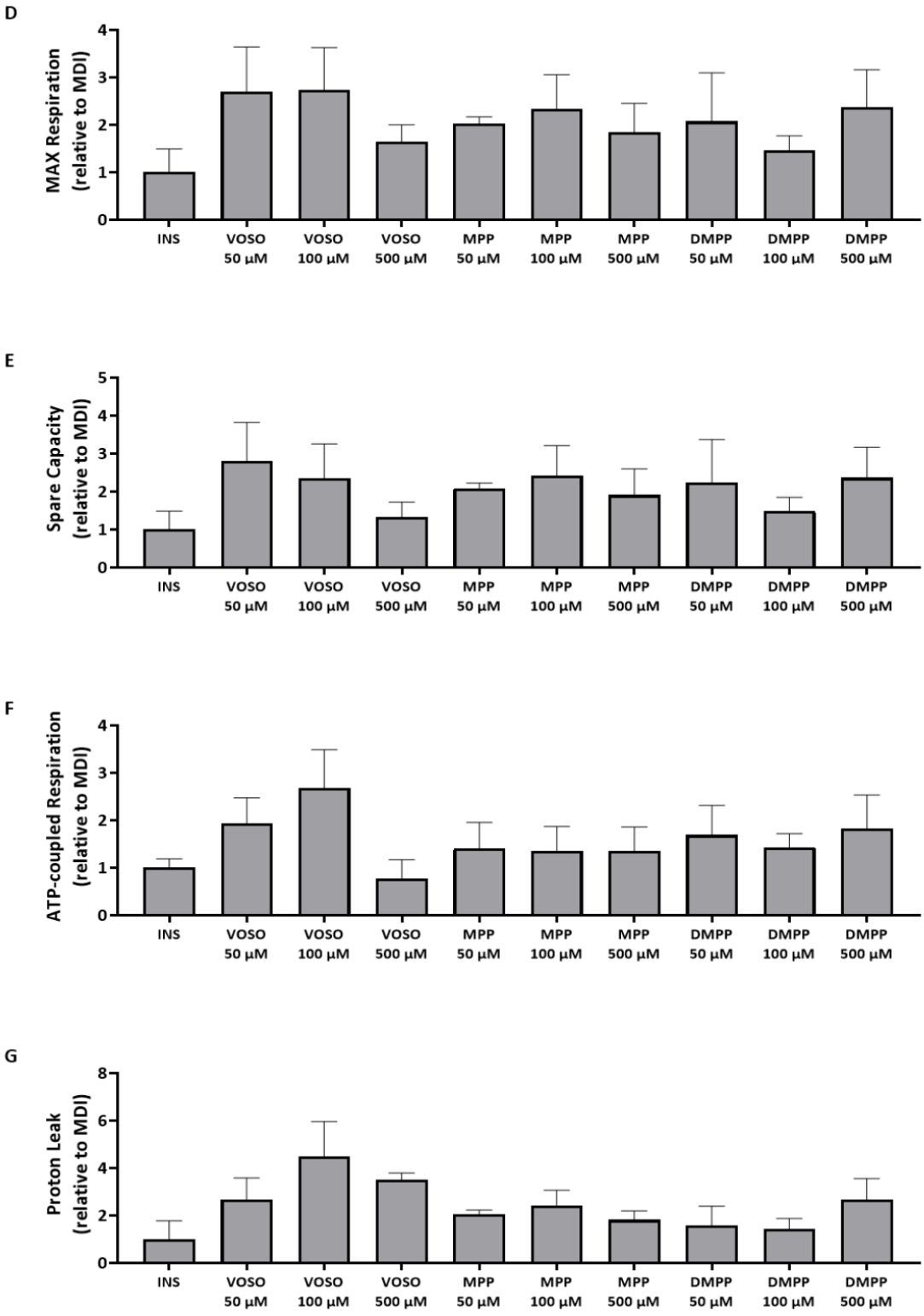


Figure 5: SeaHorse graphed parameter data. D: MAX Respiration; E: Spare Capacity; F: ATP-coupled Respiration; G: Proton Leak

4.3. Translocation of GLUT4

Figure 6 shows the results of immunofluorescence for the identification of GLUT4. DAPI staining of nuclei allows for the distinction between cytosolic and membrane GLUT4. Hence translocations of GLUT4 vesicles may be assessed in rapport to the untreated 0-hour control and the 4-hour insulin control condition.

Insulin distinctly shows higher intensity of membrane GLUT4 staining than all other conditions. From all vanadium conditions, all three concentrations of DMPP showed higher translocation then their equivalents in other compounds, particularly the 500 µM condition.

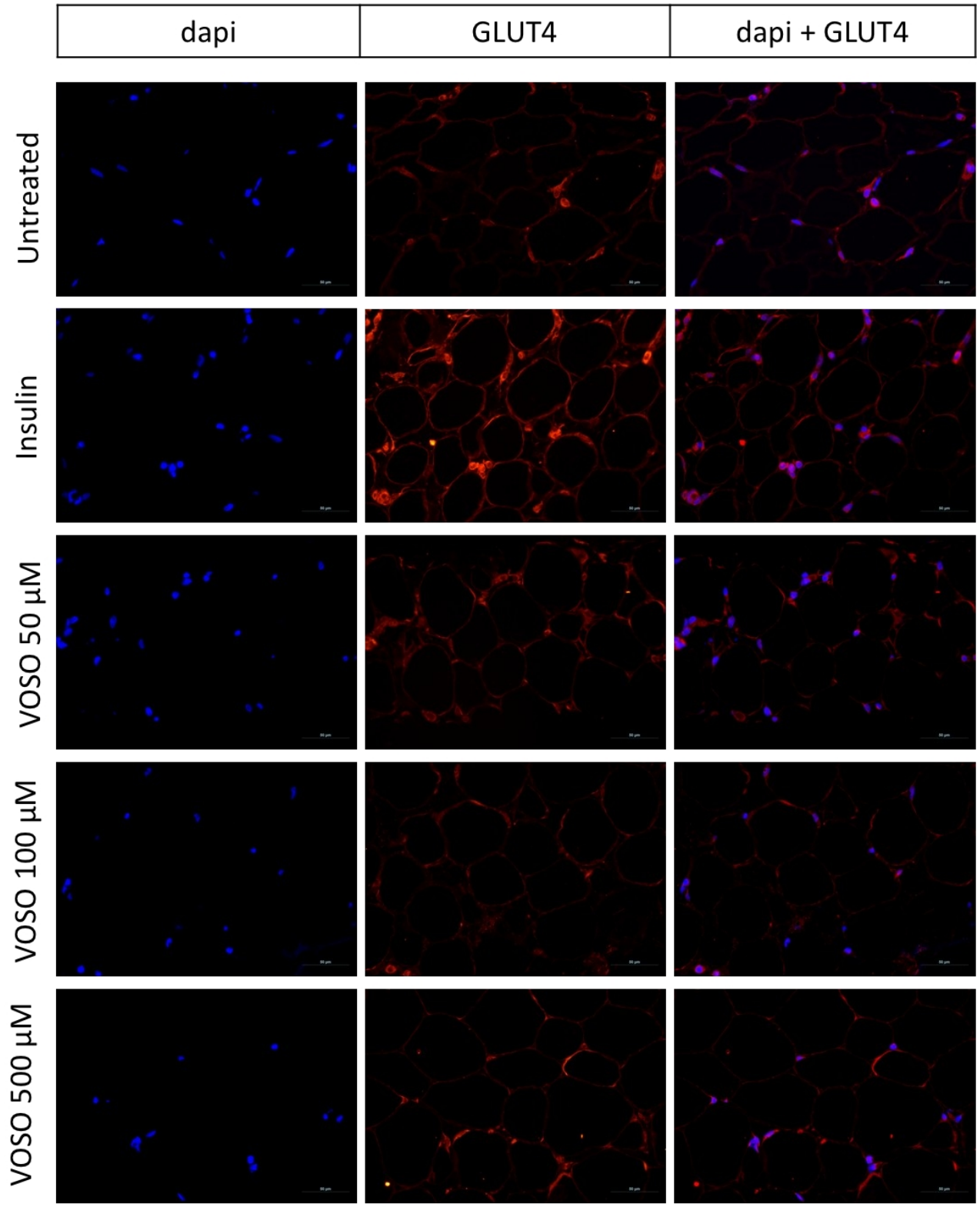


Figure 6: Immunofluorescence characterisation of GLUT4 in the various studied conditions. (continues)

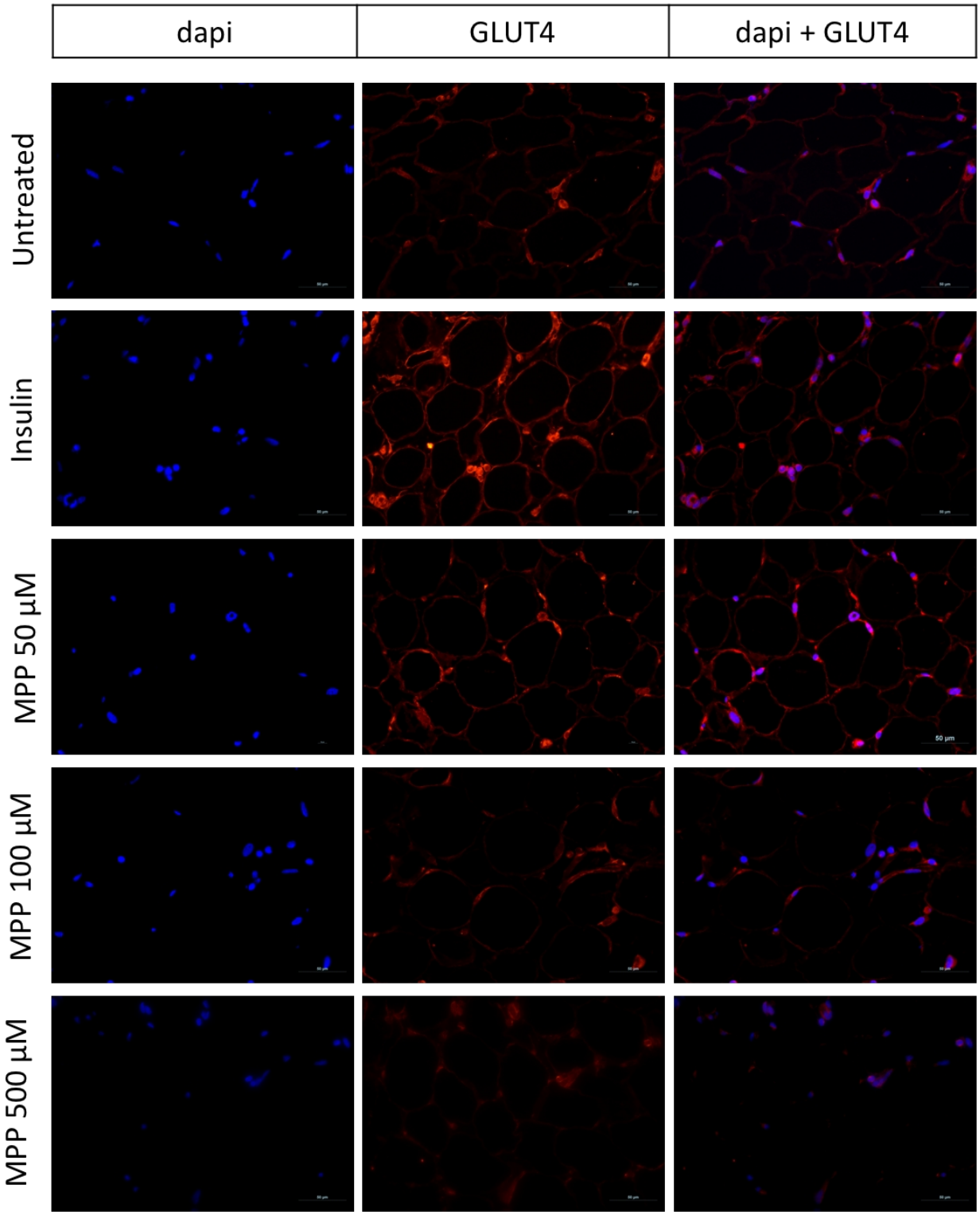


Figure 6 (continued): Immunofluorescence characterisation of GLUT4 in the various studied conditions.

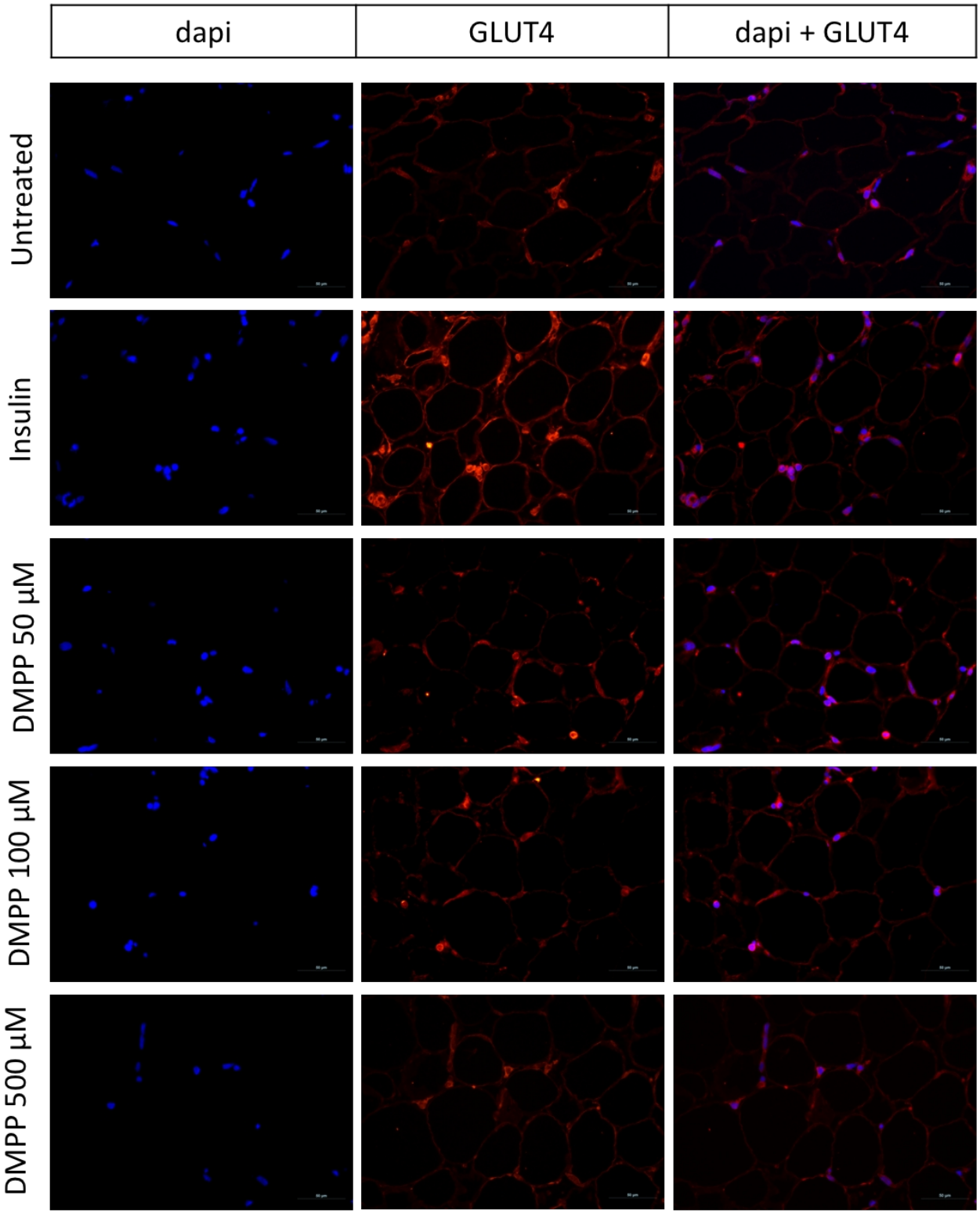


Figure 6 (continued): Immunofluorescence characterisation of GLUT4 in the various studied conditions.

4.4. Phospho-ERK Western Blot

The figure 7 below presents an image capture of the western blot for phospho-ERK in which lane and band identification are present, together with band quantification values. All lanes are normalized by total lane protein load, and expressed to the insulin condition intensity.

Except for the MPP 500 µM condition, all samples increased in phospho-ERK relative protein load, in particular VOSO 500 µM, VOSO 100 µM and MPP 50 µM.

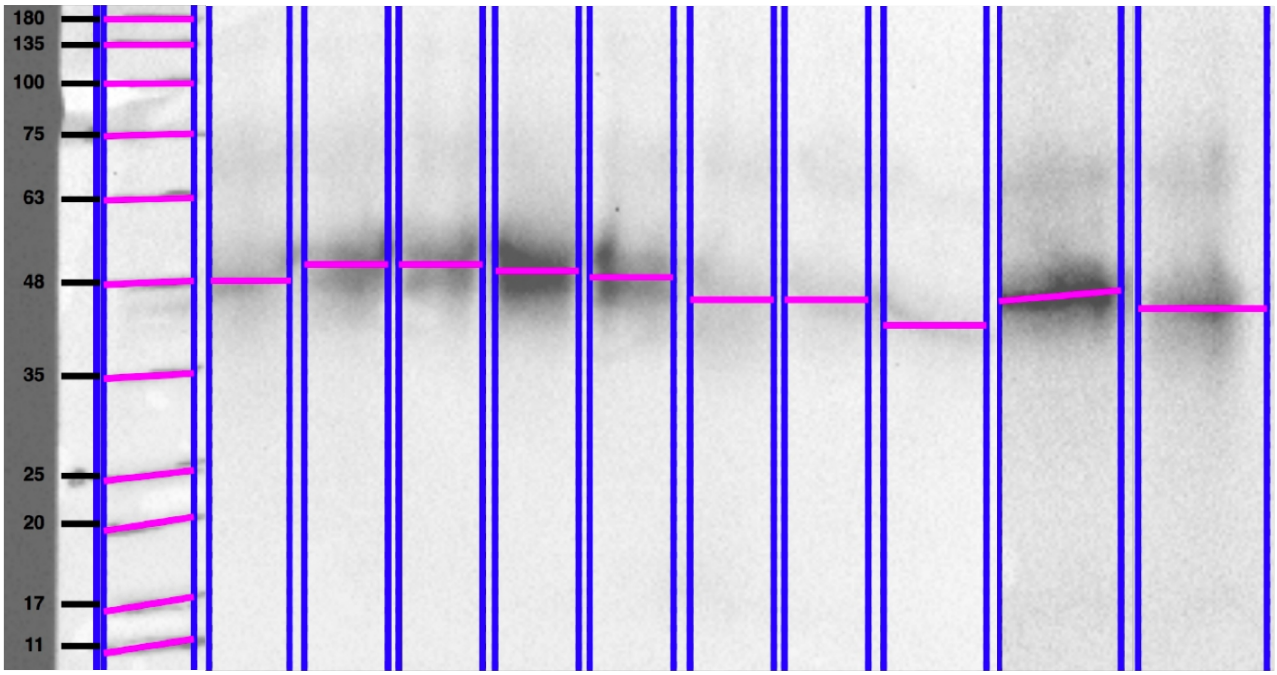
MW (kDa)	Insulin	VOSO 50 µM	VOSO 100 µM	VOSO 500 µM	MPP 50 µM	MPP 100 µM	MPP 500 µM	DMPP 50 µM	DMPP 100 µM	DMPP 500 µM
										
Band Quantification	1	2.08	3.08	4.69	3.58	1.58	0.84	1.82	2.51	1,90

Figure 7: Phospho-ERK western blot band protein quantification. Normalized by total lane protein, standardized to the INS condition.

5. Discussion

With this work we aimed to explore how two oxovanadium complexes, $VO(mpp)_2$ and $VO(dmpp)_2$ previously implicated as potential antidiabetic compounds would affect the metabolism and oxygen consumption of human visceral adipose tissue.

To address this goal, the seahorse assay was envisioned as a powerful approach to evaluate the possible metabolic and bioenergetic effects of these compounds. Together with immunofluorescence imaging of GLUT4 cytosol to membrane translocation, and quantification of phospho-ERK activation, this would aim to understand how human adipose tissue would respond.

Vanadium compounds have been implicated in oxidative stress and ROS generation in several different types of tissues such as cardiac and skeletal muscle, in liver, kidney, lung among other. (Rehder, 2020). In this study, the bioenergetics assay had some indications towards a similar tendency in adipose tissue, since the different vanadium compounds all had an apparent increase in non-mitochondrial OCR relative to controls, which is associated with oxidative stress (Chacko et al., 2014).

An increase in both total base OCR and MAX respiration for all vanadium conditions may be an indication of a higher uptake and metabolism of the energy substrates, glucose and glutamine. Consequently, this may suggest an augment in mitochondrial respiration in basal and unrestricted energy supply scenarios.

A more attentive look into the GLUT4 vesicle cytosol-membrane translocation response to vanadium compound stimuli was thought to be essential to better discern how the metabolic effects of vanadium manifest themselves mechanistically. It is has been suggested elsewhere that one of the possible ways in which vanadium acts is by irreversibly inhibiting PTP-1B (Weiss et al., 2000), is one of the main inhibitors of the main insulin signalling cascade. This would expectedly manifest itself by an increase in GLUT4 translocation and consequent glucose uptake. While glucose uptake was not directly measured in this work, by measuring membrane GLUT4 we can assess the extent to which that may be occurring. It was observed that insulin by itself was a more potent activator of this process as compared to itself in association with the vanadium compounds. The bioenergetic results showed an increased mitochondrial OCR, that suggests an early-on rapid consumption of substrate. Thus assessment of GLUT4 membrane localisation at 4 hours may not be representative of maximum treatment effect.

Nevertheless, DMPP still had a more significant membrane GLUT4 labelling when compared to the other vanadium compounds, which points to a more sustained impact in the insulin signalling pathway. However, according to our western blot results, this might not be solely due to the ERK activation pathway, since VOSO seems to produce a stronger activation of MAPK/ERK signalling without an associated translocation of GLUT4 in adipose tissue. This does not exclude the influence

of vanadium compounds through other cellular pathways such as the well described PI3K/Akt. Insulin binding to the insulin receptor leads to activation of PI3K/Akt that ultimately leads to release of GLUT4 vesicles.

As glucose metabolism is a complex and dynamic process, it would be important to further characterise the temporal profile of the insulin mimetic effect of vanadium complexes in adipose tissue metabolism. Hence, inclusion of additional timepoints for immunofluorescence characterisation of GLUT4 expression and distribution and cell signalling (not only MAPK-ERK but also encompassing PI3K-AKT) would be important to include in future studies. A more thorough normalization of phospho-ERK by total ERK expression should also be implemented. Quantitative measurement of the rate of glucose uptake using fluorescent or isotope labelled glucose would further contribute to deeper insight of metabolism dynamics in this context.

The new data generated in these studies with human samples should be substantiated with an increase in sample size for a more robust and detailed analysis of results. Further studies should also assess vanadium compounds effects in diabetic human samples.

6. Conclusions

To our knowledge we generated the first human tissue data on the influence of vanadium compounds in mitochondrial metabolism through the use of the Seahorse analyser. We observed increased oxygen consumption rates in both mitochondrial and non-mitochondrial context. This points to increased oxidative stress in adipose tissue both with the traditional inorganic salt and the more recent oxovanadium complexes while a specific effect on insulin signalling could not be attested.

More studies are needed to further validate results. These will require higher sample sizes, optimized timepoints for collection of the exposed samples as well as optimized compound concentrations, and a precise evaluation of the effect of oxidative stress due to the action of Vanadium.

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