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*EFFECTS of TNF-RELATED APOPTOSIS-INDUCING LIGAND ON AN ANIMAL MODEL of  
TYPE 2 DIABETES*

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# BACKGROUND

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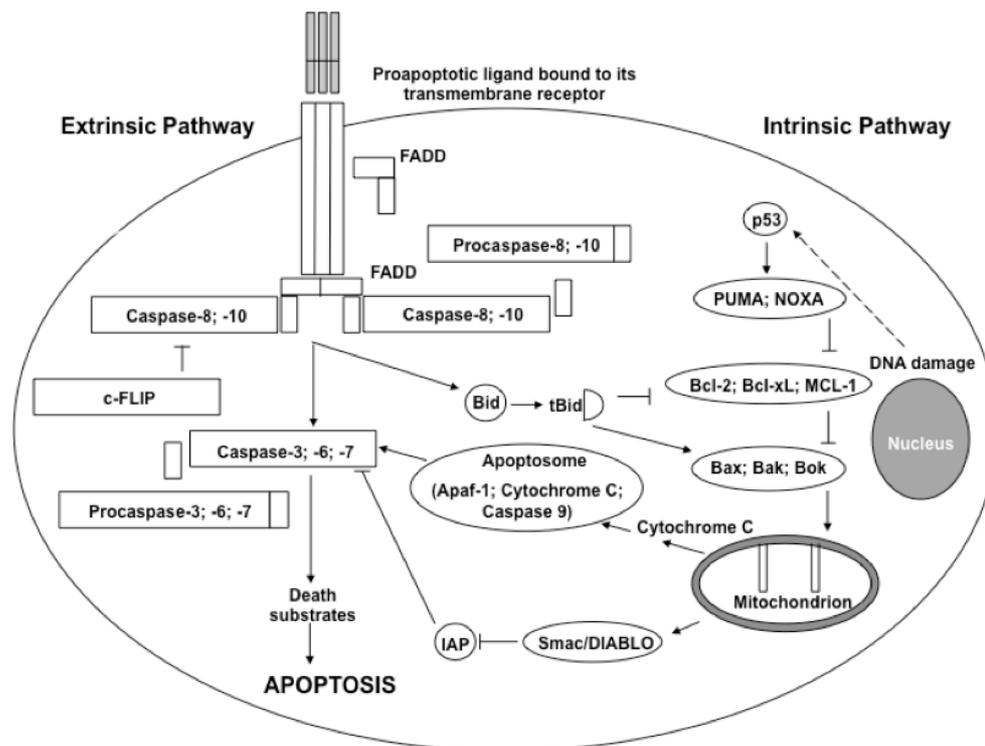
# TRAIL and cell death

## 1.1 An overview on cellular apoptosis

For a single-cell organism, life with mutations is apparently better than no life at all, but in multicellular organisms the health of the organism takes precedence over the life of an individual cell and thus, when DNA damage occurs, the cells do not attempt to continue division but instead “commit suicide” by undergoing apoptosis. Apoptosis is a process leading to cell death, whereby unnecessary or damaged cells can be eliminated. According to the source of the pro-apoptotic signal, which can be either intracellular or extracellular, two separate ways of signalling take place, both converging to the activation of effector caspases and ultimately leading to cell death. These two ways of signalling have been divided into the intrinsic pathway, triggered by an intracellular signal, such as DNA damage, and the extrinsic pathway, triggered by an extracellular signal, which usually derives from cytotoxic cells of the immune system, as in **(Figure 1)**[[Bernardi, 2012a](#)].

Briefly, the activation of the intrinsic pathway depends on the balance between intracellular pro-apoptotic and anti-apoptotic proteins that control the release of mitochondrial cytochrome C, leading to the formation of the apoptosome, caspases activation, and then cell death **(Figure 1)**. The proteins regulating the intrinsic pathway belong to the Bcl-2 family and they can be further divided into different subfamilies based on their function and structure. Some of them (e.g. Bax, Bak, and Bok) stimulate the release of cytochrome C, whereas other members (e.g. Bcl-2, Bcl-xL, and MCL-1) inhibit this process. A subfamily of these proteins is the BH3-only group, whose members share only the BH3 domain with the other proteins [[Adams, 2007](#)]. Bid is an important member of the BH3-only group since it connects the apoptotic pathways by transmitting apoptotic signals from the extrinsic to the intrinsic pathway. When pro-apoptotic molecules cause the permeabilization of the outer mitochondrial membrane, a protein called Smac/DIABLO is also released into the cytosol, which indirectly promotes apoptosis. Chai and colleagues have in fact demonstrated that the mitochondrial protein Smac/DIABLO interferes with the inhibitors of apoptosis proteins (IAP), thereby promoting the proteolytic activation of procaspase-3 [[Chai, 2000](#); [Wu, 2000](#)].

On the other hand, the activation of the extrinsic pathway depends on the binding of specific pro-apoptotic ligands, such as FasL/CD95L, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and TNF-related apoptosis-inducing ligand (TRAIL) to their transmembrane receptors (**Figure 1**). This stimulates the trimerization of the transmembrane receptors and the recruitment of Fas associated death domain (FADD). Subsequently, FADD recruits both caspase-8 and caspase-10, which undergo auto-activation by proteolytic cleavage and in turn activate caspase-3, caspase-6, and caspase-7, which execute the apoptotic program. In some cells, called type 1, the extrinsic pathway generates a signal strong enough to initiate apoptosis by itself. However, in the majority of cells, called type 2, this signal needs to be amplified to induce apoptosis [Ozoren, 2002]. This amplification can be generally achieved by a cross-talk between the extrinsic and the intrinsic pathway.



**Figure 1. Key components of the intrinsic and extrinsic apoptotic pathways**

Intrinsic pathway: DNA damage activates p53, which stimulates the intrinsic pathway by upregulating Puma and Noxa, which inhibit the anti-apoptotic Bcl-2, Bcl-xL, and MCL-1 and thus activate the pro-apoptotic Bax and Bak. Bax and Bak then stimulate the release of cytochrome C, which binds to the adaptor Apaf-1 to recruit the initiator procaspase-9 into a signalling complex termed apoptosome, which subsequently undergoes activation. Caspase-9 then generates caspase-3, caspase-6, and caspase-7. In addition to the release of cytochrome C, the permeabilisation of the mitochondrial membrane causes the release of the protein Smac/DIABLO, which promotes apoptosis by blocking the inhibitor of apoptosis proteins (IAP), which normally inhibits the activity of several caspases. Extrinsic pathway: specific extracellular homotrimers interact selectively with their specific transmembrane death receptors. Ligand binding induces receptor clustering and recruitment of Fas-associated death domain (FADD), procaspase-8, and procaspase-10, which then undergo activation and form altogether the death-inducing signalling complex (DISC). This activates caspase-3, caspase-6, and caspase-7, which then trigger apoptosis. In addition, caspase-8 cleaves Bid and truncated Bid subsequently stimulates Bax and Bak to activate the intrinsic pathway. Of note, DISC formation may be blocked by several mechanisms such as the presence of c-FLIP. The cross-talk between the extrinsic and the intrinsic pathways relies on Smac/DIABLO and truncated Bid. (Adapted from [Bernardi, 2012a](#))

## 1.2 TRAIL biology

TRAIL is one of the proteins binding to the pro-apoptotic transmembrane receptors, also called death receptors (DRs), which engage the extrinsic apoptotic pathway. TRAIL belongs to the TNF family of death-inducing ligands and was cloned on the basis of its sequence homology to TNF- $\alpha$  and FasL/CD95L [Wiley, 1995]. The percentage of identity with FasL/CD95L, TNF- $\alpha$ , lymphotoxin (Lt)- $\alpha$ , and Lt- $\beta$ , which are the most related members of the TNF family, is 28%, 23%, 23%, and 22% respectively. The gene encoding for TRAIL has been mapped to the 3q26 chromosomal region. It spans approximately 20 kb, and has five exons and four introns. In humans, TRAIL is expressed as a type II transmembrane protein of 281 amino acids, whose extracellular C-terminal domain undergoes enzymatic cleavage. Subsequently, TRAIL is released as a soluble molecule with biological activity. The cysteine residue at position 230 (Cys230) allows TRAIL to interact and assemble with other two molecules of TRAIL forming a trimeric ligand. TRAIL homotrimers are one of the pro-apoptotic receptor agonists that bind to DRs on the surface of target cells and induce apoptosis (Figure 1)

Five human receptors for TRAIL have been identified so far, which can be divided into death receptors (DRs) and decoy receptors (DcRs). DRs are TRAIL-R1 (DR4/TNFRSF10A) [Pan, 1997a] and TRAIL-R2 (DR5/TNFRSF10B) [Pan, 1997b; Sheridan, 1997; Wu, 1997; Milani, 2003], which are both type I transmembrane proteins containing an intracellular death domain that classically stimulates apoptosis upon TRAIL binding. A major contribution of our group to the biology of the TRAIL/TRAIL-R system was the discovery that when TRAIL-R1/DR4 and TRAIL-R2/DR5 are activated they not only stimulate the extrinsic apoptotic pathway, but they may also activate survival/proliferation pathways, such as nuclear factor  $\kappa$ B (NF- $\kappa$ B), ERK1/ERK2, and Akt [Harper, 2001; Secchiero, 2003; Zauli, 2005]. The second class of receptors includes the decoy receptors TRAIL-R3 (TRID/DcR1/TNFRSF10C) [Degli-Esposti, 1997a], TRAIL-R4 (DcR2/TNFRSF10D) [Degli-Esposti, 1997b; Marsters, 1997], and osteoprotegerin (OPG) [Zauli, 2009].

TRAIL is expressed on the surface of activated immune cells, such as natural killer (NK) cells, T cells, macrophages and dendritic cells, where it apparently functions as an immune effector molecule and it mediates anti-tumor cytotoxicity and immune surveillance [Di Pietro, 2004; Secchiero, 2008]. Studies on TRAIL  $-/-$  mice have demonstrated that mice without TRAIL are viable and fertile but more susceptible to tumor metastases, indicating role of TRAIL

in immune surveillance and host defence against tumor initiation and progression [[Cretney, 2002](#); [Sedger, 2002](#)]. In particular, TRAIL seems to mediate the ability of NK cells to block tumor growth and metastases development as well as some abilities of memory T cells [[Janssen, 2005](#)].

One of the unique aspects of TRAIL is that it has the ability to induce apoptosis preferentially in tumor cells, while it spares most normal cells [[Ashkenazi, 2008a](#)]. The balance between TRAIL death and decoy receptors was initially considered a possible mechanism whereby cells could negatively regulate TRAIL-induced cytotoxicity, therefore explaining TRAIL selectivity. Nevertheless, a correlation between this ratio and either protection or susceptibility to apoptosis has never been clearly demonstrated [[Lincz, 2001](#); [Wuchter, 2001](#); [Mirandola, 2004](#); [Kim, 2000](#)] and it is still under debate.

### 1.3 TNF-family members targeting the extrinsic apoptotic pathway

Agents that promote or restore apoptosis through the extrinsic pathway have emerged as a promising anti-cancer therapeutic strategy. The vast majority of mechanisms leading to apoptosis resistance and non responsiveness to anti-cancer agents involve the intrinsic apoptotic pathway, possibly because it has greater involvement in the initial elimination of oncogene-transformed cells. These mechanisms include mutations related to p53, mutations modulating the ratio between pro-apoptotic and anti-apoptotic proteins, and/or overexpression of proteins belonging to the IAP family. So, agents that promote or restore apoptosis through the extrinsic pathway, such as pro-apoptotic receptor agonists, should be able to circumvent some of the mutations leading to apoptosis resistance and therefore to overcome the non-responsiveness of tumor cells to anti-cancer therapies. For example, the activation of the extrinsic pathway induces apoptosis in tumors that are not responsive to chemotherapy because they have developed mutations of p53 [[Ravi, 2004](#)]. An additional reason that makes pro-apoptotic receptor agonists such an attractive therapeutic tool in the fight against cancer is that, given the interplay between intrinsic and extrinsic pathway, activating the extrinsic pathway may not only facilitate the outcome of conventional chemotherapies but it may also have a synergistic effect when combined to them (**Figure 1**), as many studies have demonstrated [[Nagata, 1997](#)].

Experimental evidence, however, has shown that not all the extracellular pro-apoptotic ligands could be used as anti-cancer agents, given that some of them display a low selectivity for tumors compared with normal cells [*Nagata, 1997; Tartaglia, 1992*]. For example, ever since the systemic delivery of Fas/Apo1/CD95 resulted in acute, lethal hepatotoxicity in mice this agent has been dismissed for cancer therapy [*Verbrugge, 2009; Shiraishi, 2004*]. Likewise, TNF- $\alpha$  systemic delivery caused pulmonary failure and coagulopathies as well as other symptomatic toxicities such as chills, fever, malaise, headache, myalgia, and nausea or vomiting in 13% of the patients studied. Therefore, these severe toxic effects that are elicited by FasL and TNF- $\alpha$  have precluded both ligands from use as systemic anti-cancer therapies [*Nagata, 1997; Tartaglia, 1992*].

In contrast to FasL and TNF- $\alpha$ , the pro-apoptotic ligand TRAIL, also known as Apo2L, seems to preferentially induce apoptosis in tumor cells rather than in normal cells in vitro [*Ashkenazi, 1999*]. Consistent with this, preclinical safety trials have demonstrated that this ligand does not cause toxic effects even at high doses. In particular, in their seminal study, Ashkenazi and colleagues demonstrated that the exposure of cynomolgus monkeys to recombinant human (rh)-TRAIL at 0.1-10 mg/Kg/day over 7 days did not induce detectable toxicity, whereas for comparison, TNF- $\alpha$  induced severe toxicity at 0.003 mg/Kg/day [*Marsters, 1997*]. On the basis of these properties, various agents/strategies targeting TRAIL-R1/DR4 and TRAIL-R2/DR5 have been developed. Most of these have been so far extremely well tolerated in clinical safety trial. These include: recombinant Apo2L/TRAIL, TRAIL-R1/DR4 and TRAIL-R2/DR5 monoclonal antibodies, collectively called pro-apoptotic receptor agonists (PARAs), as well as TRAIL gene-delivering strategies and small molecules binding to death receptors.

Chronologically, the first of these molecules that had been developed were recombinant versions of the soluble Apo2L/TRAIL, including a non tagged protein and versions tagged with exogenous polypeptides to improve the stabilization of the homotrimeric structure, such as oligomeric TRAIL polypeptides joined through leucine zippers called LZ-TRAIL [*Wiley, 2001*]. Ashkenazi and colleagues developed an optimized, clinical-grade version of rh-TRAIL, consisting of amino acids 114-281 of the endogenous molecule, without any added tag [*Ashkenazi, 2000*] and with only a zinc molecule located at position 230 of each subunit helping to stabilize the trimeric protein structure. Subsequently, several TRAIL-R1/DR4 and TRAIL-R2/DR5 monoclonal antibodies have been developed [*Chuntarapai, 2001; Ichikawa,*

2001; Georgakis, 2005; Ashkenazi, 2001; Salcedo, 2003; Camellia, 2006]. Among these, LBY-135 is the only chimeric, rather than fully human, monoclonal antibody against TRAIL-R2/DR5 [Li, 2008] (Table 1). Although PARAs share the same mechanism of action, the pharmacological characteristics that they display are different and this may influence their therapeutic potential. For example, the serum half-life of rh-TRAIL is 30 to 60 minutes in humans, whereas the serum half-life of the antibodies ranges from 6 to 21 days. In addition, rh-TRAIL binds not only to TRAIL-R1/DR4 and TRAIL-R2/DR5, like the monoclonal antibodies, but also to OPG, TRAIL-R3/DcR1 and TRAIL-R4/DcR2 [Wiezorek, 2010].

**Table 1. Summary of the pro-apoptotic receptor agonists**

Molecule	Developed by	Mechanism/Pharmacology
Ad5-TRAIL	—	Recombinant adenovirus encoding human Apo2L/TRAIL
AMG655 <i>Conatumumab</i>	Amgen	Human mAb targeting DR5
Apomab	Genentech	Human mAb targeting DR5
CS-1008/TRA8 <i>Tigatuzumab</i>	Daiichi Sankyo	Humanized version (CS-1008) of murine DR5-targeting antibody (TRA-8)
HGS-ETR1 <i>Mapatumumab</i>	HGS GSK	Human mAb targeting DR4
HGS-ETR2 <i>Lexatumumab</i>	HGS	Human mAb targeting DR5
LBY135	Novartis	Chimeric mAb targeting DR5
PRO1762/AMG951 <i>Dulanermin</i>	Genentech/Amgen	rhApo2L/TRAIL targeting DR4 and DR5
PRO95780	Genentech	Human mAb targeting DR5
DRAAs	—	Small molecules activating DRs

Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; DRAs, death receptors agonists/activators; HGS, Human Genome Science; GSK, Glaxo Smith Kline; mAb, monoclonal antibody; DR4, death receptor 4; DR5, death receptor 5. (Adapted from Bernardi, 2012a).

## 1.4 Preclinical and clinical data on the effects of TRAIL and TRAIL agonists

The first preclinical data for the soluble, non tagged rh-TRAIL were published in 1999 by Ashkenazi and colleagues [Ashkenazi, 1999], demonstrating that this molecule induced apoptosis in cell lines derived from human colon, lung, breast, central nervous system, kidney and skin cancer, while it spared normal cells. Following studies showed that rh-TRAIL was able to induce apoptosis in other malignant cell lines derived from prostate cancer [Yu, 2000], thyroid cancer [Mitsiades, 2000], leukemia [Yao, 2007], multiple myeloma [Mitsiades, 2001] and non-Hodgkin's lymphoma (NHL) [Daniel, 2007]. Consistent with these data obtained in vitro, TRAIL displayed a marked anti-tumor activity in vivo, in models of mouse xenografts of human colon or lung cancer, multiple myeloma, NHL, and glioma. Subsequently, the preclinical studies on the efficacy of TRAIL-R1/DR4 and TRAIL-R2/DR5 demonstrated that also these monoclonal antibodies were able to induce apoptosis in a wide variety of tumor cell lines and primary tumor explants [Ichikawa, 2001; Ashkenazi, 2008b].

Based on these promising preclinical results, the safety and the efficacy of pro-apoptotic receptor agonists have been evaluated in phase I and II clinical trials, as summarized in **Table 2** [Herbst, 2010a; Soria, 2010; Tolcher, 2007; Hotte, 2008; Leong, 2009; Mom, 2009; Younes, 2010; Trarbach, 2010; Greco, 2008; Herbst, 2010b; Doi, 2011; Wakelee, 2010; Plummer, 2007; Camidge, 2010; Forero-Torres, 2010]. Overall, these studies have demonstrated that the pro-apoptotic receptor agonists are safe and well tolerated. These works have given ground for additional trials, some still ongoing [Ashkenazi, 2008c]. Focusing on rh-TRAIL, two main trials have been conducted so far. In the first one, which was a phase I, open-label, and dose-escalation study, patients with advanced cancer were treated with rh-TRAIL at a dose ranging from 0.5 to 30mg/kg/day. Doses were given daily for 5 days, every 3 weeks. A total of 71 patients were treated, among whom only 2 drug-related dose-limiting adverse events were observed and a maximum tolerated dose was not reached during the dose escalation phase. In this study, rh-TRAIL demonstrated linear pharmacokinetic, with an estimated effective half-life of half an hour. Notably, one patient with chondrosarcoma exhibited a partial response with >80% tumor regression [Herbst, 2010a]. The second one was a phase Ib study in relapsed low-grade NHL and non-small-cell lung carcinoma (NSCLC) [Soria, 2010]. In NSCLC, the combination of rh-TRAIL with carboplatin, paclitaxel and bevacizumab showed a 70% overall tumor response rate with 1 complete and 13 partial responses in the 18 patients treated.

**Table 2. Development status of some of the pro-apoptotic receptor agonists**

Phase/Treatment	Patients	Results
<b>Conatumumab:</b>		
Phase Ia-Monotherapy	Advanced solid tumors (n=37)	No DTL; no MTD up to 20 mg/kg Q2W; 1 PR in NSCLC
Phase Ia-Monotherapy	Advanced solid tumors (n=18)	No DTL; no MTD up to 20 mg/kg Q2W
<b>Dulanermin:</b>		
Phase Ia-Monotherapy	Advanced solid tumors or NHL (n=71)	2 DLTs; no MTD up to 15mg/kg/d for 5d Q3W; 1 PR in chondrosarcoma
Phase Ib-+PAC, CAR and BEV	Stage IIIb/IV or recurrent NSCLC (n=24)	No DLT; no MTD up to 20 mg/kg/d for 2d Q3W; 1 CR; 13 PRs
<b>Lexatumumab:</b>		
Phase Ia-Monotherapy	Advanced solid tumors (n=37)	3 DTLs; MTD at 10mg/kg Q3W; no objective responses
Phase Ia-Monotherapy	Advanced solid tumors (n=31)	1 DTLs; no MTD at 10mg/kg Q2W; no objectives responses
<b>Mapatumumab:</b>		
Phase Ia-Monotherapy	Advanced solid tumors (n=49)	3 DTLs; no MTD up to 10 mg/kg Q2W; no objective responses
Phase Ia-Monotherapy	Advanced solid tumors (n=41)	No DTL; no MTD up to 20mg/kg Q4W; no objective responses
Phase Ib-+PAC and CAR	Advanced solid tumors (n=27)	2 DTLs; no MTD up to 20mg/kg Q3W; 5 PRs
Phase Ib-+GEM and CIS	Advanced solid tumors (n=49)	5 DTLs; no MTD up to 30mg/kg Q3W; 12 PRs
Phase II-Monotherapy	Relapsed/refractory NHL (n=40)	No DTL; no MTD up to 10mg/kg Q3W; 2CRs 1 PR
Phase II-Monotherapy	Relapsed/refrectory CRC (n=38)	No DTLs; no MTD up to 20mg/kg Q2W; no objective responses
Phase II-Monotherapy	Relapsed/refractory NSCLC (n=32)	No DTLs; no MTD up to 10mg/kg Q3W; no objective responses
<b>PRO95780:</b>		
Phase Ia-Monotherapy	Advanced solid tumors or NHL (n=50)	4 DTLs; no MTD up to 20mg/kg Q2W; no objective responses
<b>Tigatuzumab:</b>		
Phase Ia-Monotherapy	Relapsed/refractory solid tumors/NHL (n=17)	No DTL; no MTD up to 8mg/kg QW; no objectives responses

Abbreviations: PAC, paclitaxel; CAR, carboplatin; BEV, bevacizumab; GEM, gemcitabine; CIS, cisplatin; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; CRC, colorectal cancer; DLT, dose-limiting toxicity; MTD, maximum tolerated dose; QW, once per week; Q2W, every 2 weeks; Q3W, every 3 weeks; Q4W, every 4 weeks; PR, partial response; CR, complete response. (Adapted from [Bernardi, 2012a](#))

## 1.5 rh-TRAIL-based combinational therapy

A large number of studies have revealed that combining TRAIL with either traditional or novel anti-cancer agents may reverse the resistance to monotherapy. Interestingly, it seems that low concentrations of chemotherapeutics can enhance the sensitivity to TRAIL or the TRAIL receptor antibody HGS-ETR1 by accumulating the TRAIL-R1/DR4 at the cell surface [Jin, 2007]. On the other hand, pre-treatment with chemotherapeutic agents of Bax<sup>-/-</sup> human colon cancer cells that are resistant to TRAIL has been shown to restore TRAIL sensitivity. For TRAIL-based combinational therapy the agents/strategies that have been tested are listed on (Table 3) [Ravi, 2004; Frese, 2002; Jin, 2004; Hylander, 2005; Vignati, 2002; Cuello, 2001; Mizutani, 1999; Brooks, 2005; Johnson, 2003; Zhu, 2005; Ricci, 2007; Kim, 2008; Meng, 2007; Rosato, 2007; Zangemeister-Wittke, 2003; Ray, 2005; Li, 2004a; Arnt, 2002; Adams, 2007; Chai, 2000; Fulda, 2002; Li, 2004b; Guo, 2004; Maier, 2011; Croce, 2008; Garofalo, 2009]

**Table 3. Summary of anti-cancer agents studied in combination with rh-TRAIL**

Mechanism of action	Agent	Experimental model
<b>Cytotoxicity by traditional chemotherapies</b>	Camptothecin, 5-fluorouracil, irinotecan, cisplatin, paclitaxel, doxorubicin, carboplatin, gemcitabine, adriamycin	In vitro
	Paclitaxel, carboplatin, bevacizumab, cisplatin, gemcitabine	In vivo
<b>Proteasome inhibition</b>	Bortezomib	In vitro
<b>Kinase inhibition</b>	Sorafenib	In vitro
<b>Bcl-2 inhibition</b>	Genasense	In vitro
	BH3I-2'	In vitro
<b>IAP inhibition</b>	Smac peptides	In vitro and in vivo
<b>HDACs inhibition</b>	TSA, SAHA, LAQ824	In vitro
<b>miRNAs modulation</b>	Strategies increasing miR-212 and strategies decreasing miR-221 and miR-222	In vitro

Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; BH3I, BH3 inhibitor; IAP, inhibitor of apoptosis proteins; HDACs, histone deacetylases; miRNA and miR, microRNA. (Adapted from Bernardi, 2012a)

# Potential role of TRAIL in the management of autoimmune diabetes

## 2.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disease whose incidence has been steadily increasing worldwide since the middle of the 20<sup>th</sup> Century. Recently, the increase in T1DM incidence has been even faster than before and it is expected that the number of new cases diagnosed at or before 14 years of age will double in the next 15 years and the age of onset will be younger (0-4 years) [*Harjutsalo, 2008*]. It has been shown that T1DM results from an autoimmune  $\beta$ -cell destruction and when 70-80% of the  $\beta$ -cell mass has been destroyed the residual functional  $\beta$ -cells are insufficient in number to maintain glucose tolerance [*Van Belle, 2011*]. At this stage, the lack of insulin leads to hyperglycemia and T1DM becomes clinically apparent [*Van Belle, 2011*].

T1DM therapy is based on the use of insulin. Despite the improvements in insulin replacement regimens and devices, insulin remains a treatment that heavily affects patients' quality of life and retains some important side effects, such as hypoglycemic events, seizures and coma. Moreover, T1DM is still associated with substantial morbidity and mortality. Consequently, during the past 20 years, a lot of interest has been focused on developing new strategies that aim to prevent, delay, and treat T1DM. These strategies are based on targeting the immune system [*Bernardi, 2012b*]. In contrast to the broadly immunosuppressive agents that were used initially, failed to produce lasting remission, and were associated with multiple long-term side effects, new rationally designed agents have shown to possess the characteristics to successfully prevent or delay T1DM development [*Van Belle, 2011; Feutren, 1986; Dupré, 1988; Raz, 2001; Herold, 2005; Lugdvisson, 2012; Hu, 2007*]. TRAIL is one of these agents, given that by regulating the homeostasis of the immune system it seems to delay T1DM natural history without causing adverse effects [*Di Pietro, 2004; Secchiero, 2008*].

## 2.2 Autoimmunity in T1DM

T1DM is an autoimmune disease in which CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes infiltrate the Islets of Langerhans resulting in a cell-mediated islets destruction, insulin deficiency and hyperglycemia. The concept that T1DM pathogenesis is immune-mediated dates back to the

sixties, when Burnet and Mackay included T1DM among several autoimmune diseases. Two years later, Gepts and colleagues found that the key pancreatic morphological feature in recent-onset T1DM was an insulinitis, suggesting that it is the immune system that is involved in the pathogenesis of the disease [Gepts, 1965]. Then, in 1974 the group of Nerup demonstrated that T1DM is a cell-mediated autoimmune disease by incubating porcine islets with peripheral blood mononuclear cells of diabetic patients and reporting cell migration inhibition in diabetics as compared to normal controls [Nerup, 1974].

The current view on the human immunology of T1DM could be summarized as follows: T1DM is a T cell-mediated disease. T cells are present in the islets, peri-pancreatic lymphnodes and systemic circulation of T1DM patients. These cells are mostly CD8<sup>+</sup> lymphocytes, with only few macrophages and no T regulatory (Treg) cells, as to say that the distribution of T cell subsets is also abnormal [Buschard, 1980] and that circulating Treg cells display defective responsiveness [Roncarolo, 2007]. The importance of Treg cells in the maintenance of self-tolerance and immune homeostasis has been clearly shown by the development of T1DM and other polyendocrinopathies in patients with immune-dysregulation polyendocrinopathy enteropathy X-linked syndrome, caused by impairment of Treg cell-mediated peripheral self-tolerance [Sakaguchi, 2012]. On the other hand, the report that a patient with X-linked agammaglobulinemia developed T1DM diabetes shows that neither autoantibodies nor B cell function seem to be critically involved in the pathogenesis of this disease [Martin, 2001]. Although the delivery of rituximab, which is an anti-B cell antibody, protected NOD mice against T1DM development during the early stages of the disease [Hu, 2007], the same treatment did not change the disease course in early onset T1DM patients [Pescovitz, 2009], suggesting once again that cell-mediated autoimmunity in T1DM corresponds to a T cell-mediated response.

As for the natural history of the disease, islet inflammation or insulinitis generally precedes the onset of T1DM [Roep, 1986] and autoantibodies are usually detectable at this stage [Eisenbarth, 1986]. These autoantibodies are directed against several molecules of  $\beta$ -cells, such as insulin, insulinoma-associated antigen (IA)-2, 65-kD isoform of glutamic acid decarboxylase (GAD-65),  $\beta$ -cell-specific zinc transporter (Znt8), and islet cell autoantibodies (ICA)-512. The role and/or significance of these autoantibodies is not entirely clear, given that fetal exposure to GAD-65 and/or IA-2 autoantibodies may protect against diabetes [Bottazzo, 1978]. Proinsulin, GAD-65, IA-2 and Znt8 are not only the major autoantibody targets but also

the main epitopes activating autoreactive T cells [*Koezwarra, 2004*]. Once T cells have been activated by the recognition of islet autoantigens via MHC class I [*Di Lorenzo, 2007*] and II,  $\beta$ -cells are destroyed either by CD8+ lymphocytes through the release of cytotoxic granules containing perforin and granzyme, or by both CD8+ and CD4+ lymphocytes through Fas and Fas-ligand-dependent interactions or the release of IL-1 $\beta$ , TNF- $\alpha$ ,  $\gamma$ -interferon (IFN- $\gamma$ ) [*Hamilton-Williams, 2003*].

### 2.3 Immunosuppressive strategies in T1DM or from cause to cure

Given that autoimmunity appears to be the main effector mechanism in T1DM, several immunosuppressive agents have been studied as potential therapies for patients with new-onset T1DM. The first agents studied were broadly immunosuppressive drugs including cyclosporine, nicotinamide, anti-oxidant agents, heat shock proteins and anti-CD3 [*Feutren, 1986; Dupré, 1988; Raz, 2001; Herold, 2005*]. Unfortunately, most attempts either achieved minimal benefits or the adverse effects outweighed their benefits. One of the main side effects of cyclosporine was, for example, the induction of nephropathy [*Feutren, 1986; Dupré, 1988*]. So, the scientific interest moved to antigen-specific interventions, which are more targeted forms of immunosuppression, aiming at inducing selective immunological tolerance by the systemic delivery of the autoantigens involved in T1DM. The delivery of these autoantigens, such as GAD-65 and insulin, should in fact induce Treg cell responses (active tolerance) and anergize autoreactive T cells (passive tolerance). Consistent with this rationale, in one of these studies GAD-65 treatment displayed a short protective effect on C-peptide secretion in T1DM patients, who had been treated less than 6 months after diagnosis [*Ludvigsson, 2008*]. Nevertheless, the results coming from a more recent trial did not confirm the efficacy of such a treatment in the long-term, as GAD-65 delivery failed to ameliorate T1DM natural history over 15 months [*Ludvigsson, 2012*].

Beside these immunosuppressive strategies, another therapeutic strategy against T1DM, which aims at curing this disease rather than just treating it, is islet transplantation. Islet transplantation has been shown to successfully restore endogenous insulin production and glycemic stability in subjects with T1DM and instable control [*Ricordi, 2003*]. In particular, the islet transplantation performed according to the Edmonton protocol has achieved reproducible insulin independence, with more than 80% of recipients still insulin-free at 1 year after the

procedure [*Shapiro, 2006*]. Currently available data suggest that medium- to long-term results are still not achievable mainly because of the high frequency of non-functioning grafts and secondary graft failure. Unfortunately, the methods that have been devised so far to protect  $\beta$ -cells from immune destruction have only been able to delay, without preventing, the failure of the transplants and the consequent need of resuming the administration of insulin. Moreover all the adverse effects of long-term general immunosuppression and late graft loss overall represent an obstacle for any successful transplantation [*Battaglia, 2006*].

## 2.4 TRAIL, autoimmune diseases, and T1DM

A large amount of data has pointed out the ability of TRAIL to act as an immune system modulator [*Di Pietro, 2004; Secchiero, 2008*]. TRAIL best-known function is the induction of apoptosis, which occurs through the activation of the extrinsic apoptotic pathway, triggered by the binding of TRAIL to DRs, as discussed before. Beside its pro-apoptotic effect, TRAIL is also able to activate pathways involved in cell survival. It has in fact been demonstrated that TRAIL is able to stimulate the survival and proliferation of both neoplastic and non-neoplastic cells [*Lancaster, 2003; Ehrhardt, 2003; Secchiero, 2004; Morel, 2005*]. The TRAIL-mediated pro-survival effect seems to be due to the recruitment of adaptor molecules that interact directly with DRs death domains. This would block the progression of the pro-apoptotic signal. In the meantime, TRAIL would be able to engage other intracellular pathways and mediators, including NF- $\kappa$ B, JNK, MAPK/ERK and Akt, leading to subsequent transcription of survival genes [*Morel, 2005; Secchiero, 2004*]. TRAIL ability of activating diametrically opposed pathways may actually be ascribed to cell responsiveness, rather than to the ligand properties. Cell responsiveness to TRAIL could result from the balance between death and decoy receptors expressed on the cell surface [*Lincz, 2001; Wutcher, 2001; Mirandola, 2004; Kim, 2000*], which in turn could come down to the cellular incorporation of specific intracellular proteins into “lipid rafts” [*Hunter, 2006; Song, 2007*]. Lipid rafts are platforms consisting of a dynamic pool of cholesterol and sphingolipids, which recruit signalling molecules including cell surface receptors. Independent studies have documented a redistribution of TRAIL receptors and DISC components from “non-rafts” into lipid rafts suggesting that this event could switch apoptosis instead of survival [*Song, 2007*]. However, a correlation between TRAIL receptors balance (i.e., death/decoy receptor ratio) and protection versus susceptibility to apoptosis has never been clearly demonstrated and it is still under debate.

Going back to T1DM, TRAIL is expressed on the surface of activated immune cells, such as NK cells, T cells, macrophages and dendritic cells, where it apparently mediates immune surveillance, particularly against hematological malignancies and autoimmune diseases [Secchiero, 2008]. Studies on TRAIL -/- mice demonstrate that mice without TRAIL are viable and fertile, but more susceptible to autoimmune arthritis, experimental autoimmune encephalomyelitis and autoimmune diabetes, indicating a clear role for TRAIL in immune regulation. In addition, TRAIL involvement in immune surveillance has also been proved by the acceleration of experimental autoimmune encephalomyelitis (EAE) and autoimmune arthritis that followed TRAIL blockade [Cretney, 2002; Sedger, 2002]. Interestingly, in the models of EAE TRAIL-deficient mice display significantly lower Treg cells than wild-type mice, as to say that TRAIL not only inhibits autoreactive T cells but also promotes Treg cells [Ikeda, 2010].

Lamhamedi-Cherradi and colleagues are the first who have demonstrated that either blockade or TRAIL deficiency exacerbates T1DM [Lamhamedi-Cherradi, 2003]. The first animal model consisted of NOD mice injected with a soluble TRAIL receptor (sDR5/TRAIL-R2) in order to block TRAIL functions. In the mice injected with sDR5/TRAIL-R2, T1DM incidence increased significantly and its onset was also significantly accelerated with respect to the control group. In addition, the blockade of TRAIL by sDR5/TRAIL-R2 led to greater pancreatic inflammation as well as to enhanced cellular and humoral immune responses, with an increase in T cells proliferation and pro-inflammatory response as well as higher levels of anti-GAD-65 [Lamhamedi-Cherradi, 2003]. The second animal model consisted of TRAIL -/- mice injected with streptozotocin, which is a drug that destroys  $\beta$ -cells and therefore cause diabetes. The findings observed in the diabetic TRAIL -/- mice recapitulated those observed in the sDR5 treated NOD mice, since the incidence of T1DM increased significantly and its onset was significantly accelerated. Consistent with these observations, the administration of recombinant soluble TRAIL in streptozotocin-injected mice preserved pancreatic islets and significantly ameliorated the severity of T1DM, by lowering glucose levels [Zauli, 2010].

## 2.5 Possible mechanisms whereby TRAIL protects against T1DM

Having said that, the molecular mechanisms whereby TRAIL blockade exacerbates T1DM and, on the contrary, its delivery ameliorates the natural history of this disease, have

only partly been clarified. One of the studies addressing this issue showed that TRAIL suppressed the proliferation of autoreactive T cells isolated from diabetic NOD mice [Mi, 2003]. This effect was explained by the finding that TRAIL up-regulated p27 expression and simultaneously inhibited IL-2 production by autoreactive T cells. With respect to this issue, it has indeed been shown that the up-regulation of cdk inhibitors, such as p27, which sequester cyclin D2-cdk4 and cyclin E-cdk2 complexes, stops the progression of the cell cycle, preventing the progression of T cells through the G1 restriction point of the cell cycle, and therefore anergizing autoreactive T cells [Mi, 2003]. On the other hand, it has been shown that the cell exposure to IL-2 promotes the degradation of p27 and the entry into S phase [Appelman, 2001]; therefore IL-2 inhibition by TRAIL could be an additional mechanism whereby TRAIL anergizes diabetogenic T cells.

Recent works suggest that beside T cells anergy TRAIL could also promote  $\beta$  cell survival. *In vitro* experiments performed on rat insulinoma cells (INS-1), which are used as a model for the study of pancreatic  $\beta$ -cells, have shown that exposure to soluble TRAIL does not affect  $\beta$ -cell viability, but, by promoting the activation of NF- $\kappa$ B, it upregulates the expression of its decoy receptor TRAIL-R3 (DcR1), which in turns should prevent apoptosis [Kang, 2010a]. In this context, the selective increase of TRAIL and DcR1 expression in the pancreatic islets in NOD mice, which occurs in streptozotocin-induced T1DM, has been proposed as part of a defensive strategy of the  $\beta$ -cell against infiltrating leukocytes [Dirice, 2011].

Kang and colleagues have further investigated TRAIL protective effects in T1DM animal models, demonstrating that they could be partly attributed to the elevation of tissue inhibitor of metalloproteinase-1 (TIMP-1) levels that follows TRAIL delivery [Kang, 2010b]. *In vivo* experiments have in fact demonstrated that TRAIL-induced TIMP-1 elevation markedly reduced MMP-9 pancreatic activity, thus protecting against T1DM development/progression [Kang, 2010b], given that circulating MMP-9 is higher in diabetic patients and that MMP-9 cleaves insulin [Maxwell, 2001; Descamps, 2004; Xue, 2005]. The *in vivo* data are further supported by *in vitro* data showing that the addition of TIMP-1 significantly reduces INS-1 cells death [Kang, 2010b], consistent with a previous report showing that TIMP-1 prevents cytokine-mediated dysfunction and cytotoxicity in pancreatic islets and  $\beta$ -cell [Han, 2001].

## 2.6 TRAIL and diabetic complications

It is well known that T1DM morbidity and mortality are primarily due to diabetic complications. Diabetic complications are divided into macrovascular and microvascular and they include acute myocardial infarction, stroke, peripheral artery disease, diabetic nephropathy, retinopathy and neuropathy [Calcutt, 2009]. Experimental works suggest that TRAIL may protect against diabetic complications. With respect to cardiovascular diseases, our group has demonstrated that TRAIL delivery significantly reduced the development of atherosclerotic lesions and contributed to plaque stabilization by decreasing the number of infiltrating macrophages and increasing the number of vascular smooth muscle cells in an animal model of T1DM [Secchiero, 2006]. The concept that TRAIL might protect against cardiovascular diseases, is supported by the finding that circulating TRAIL is significantly reduced in different cohorts of patients with acute coronary syndrome and heart failure with respect to patients with stable angina or healthy coronary arteries [Michowitz, 2005; Schoppet, 2006; Secchiero, 2009]. In addition to the anti-atherosclerotic properties of TRAIL we have also demonstrated that TRAIL delivery protects against myocardial fibrosis and cell apoptosis in an animal model of diabetic cardiomyopathy [Toffoli, 2012], which corresponds to the stage in which ventricular dysfunction develops in diabetic patients in the absence of coronary atherosclerosis and hypertension.

In contrast to what has been found in experimental models of cardiovascular diseases, TRAIL unfortunately does not seem to protect against diabetic microvascular complications. In an animal model of diabetic nephropathy Lorz and colleagues [Lorz, 2008] found that TRAIL gene and protein expression was significantly increased in both glomeruli and tubuli of diabetic kidneys. In following *in vitro* experiments it was found that TRAIL actually induced tubular cell apoptosis, which was enhanced by the concomitant presence of pro-inflammatory cytokines and high glucose levels. This would suggest that TRAIL could play an important role in the progression of diabetic nephropathy. The concept that TRAIL may play a role in the natural history of diabetic complications is further supported by the observation that patients with proliferative diabetic retinopathy exhibited lower levels of TRAIL in the conjunctival sac fluid with respect to patients with non-proliferative diabetic retinopathy or without diabetic retinopathy [Secchiero, 2011].

## High-fat diet and type 2 diabetes mellitus

### 3.1 Obesity and type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) represents a heterogeneous group of disorders characterized by insulin resistance and impaired insulin secretion and defined by a raised fasting or post-challenge blood glucose. T2DM incidence is increasing to the point that it is reaching epidemic proportions. Recent studies show that T2DM prevalence is strongly associated with overall obesity and central obesity, mainly due to unhealthy changes in lifestyle [Yang, 2010]. It has in fact been shown that obesity is an independent predictor of insulin resistance and T2DM [Collins, 2011] and that weight loss prevents it [Tuomilhto, 2011]. Among the multiple mechanisms linking obesity to diabetes, inflammation is a common feature that has been implicated in the pathophysiology of both diseases.

### 3.2 Lipotoxicity and T2DM

It has been argued that another mechanism explaining insulin resistance and compromised  $\beta$ -cell function in T2DM is a breakdown in lipids dynamics. This is often reflected by elevated levels of circulating free fatty acids (FFA) and triglycerides, together with excessive deposition of fat in various extra-adipose tissues including the muscle bed and the  $\beta$ -cells. Increased tissue levels of FFA have been shown to cause the  $\beta$ -cell abnormalities of non-diabetic obesity and ultimately result in obesity-dependent diabetes [Unger, 2011]. A western diet that is high in fat will therefore result in loss of  $\beta$ -cells, by pancreatic FFA deposition that in turn promotes lipoapoptosis as well as peripheral glucose intolerance due to lipid excess in skeletal muscle.

### 3.3 Animal models of T2DM

T2DM animal models are likely to be as complex and heterogeneous as the human condition. [Chatzigeorgiou, 2009] However, since obesity is the major environmental factor predisposing to T2DM (although 2/3 of obese subjects do not become diabetics), the ability of an animal model to develop firstly obesity and then T2DM is one of the most important criteria for selecting a model. T2DM murine models are summarized on **Table 4**.

### 3.4 High-fat feeding as a model for the study of T2DM

Rodent models, both genetic and dietary (Table 4), are commonly used to examine the mechanisms underlying the development of insulin resistance in the setting of obesity. Many of these animal models, such as the high-fat fed rodents and those with an absence/impairment of leptin signalling, are based on the fact that an over supply of lipids has been identified as a key factor leading to obesity and insulin resistance [Turner, 2007]. Male mice C57Bl6 8 weeks old, high-fat fed for 12 weeks become obese and develop a mild form of T2DM compared to control mice.

In particular we have found [Bernardi, submitted data]: (i) fat deposition; (ii) hyperglycemia, hyperinsulinemia and insulin resistance (**Figure 2**); (iii) low-grade inflammation (**Figure 3**). In conclusion, high-fat fed C57Bl6 mice develop a form of obesity-induced T2DM presumably caused by an accumulation of lipids leading to dysfunction of pancreatic islets and peripheral organs as well as adipose tissue enlargement and low-grade inflammation.

**Table 4. Rodent models of T2DM**

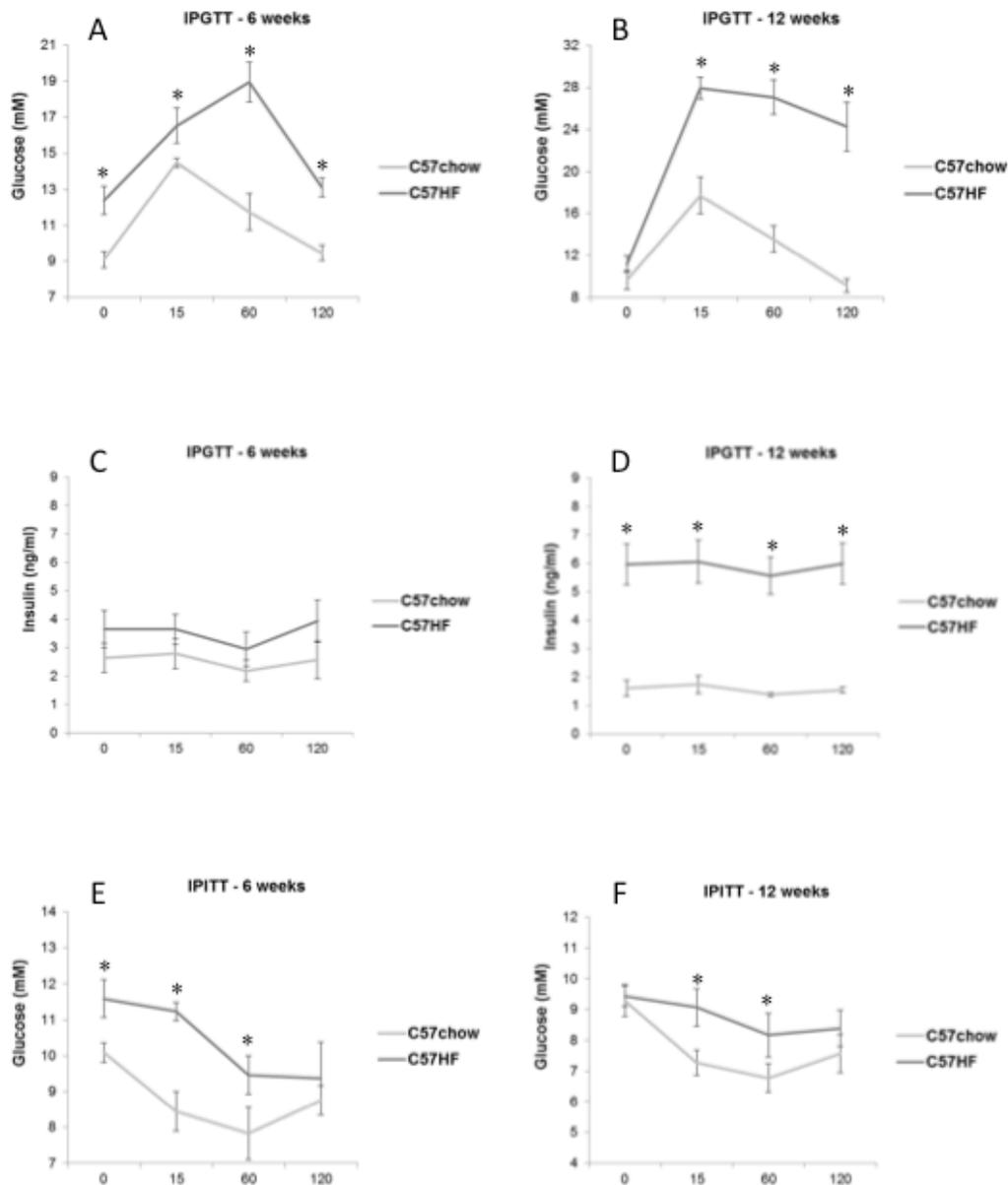
Type of animal model	Obese	Non-obese
<b>Spontaneous or genetically derived models</b>	ob/ob mouse db/db mouse Zucker (fa/fa) fatty rat KK (Kuo Kondo) mouse KK/A <sup>y</sup> (yellow KK obese) mouse NZO (New Zealand obese) mouse NONcNZO10 mouse OLETF (Otsuka Long Evans Tokushima fatty) rat ZDF (Zucker diabetic fatty) rat JCR/LA-cp (James C Russell/LA corpulent) rat SHR/N-cp (spontaneously hypertensive rat/NIH-corpulent) rat M16 mouse TSOD (Tsumara Suzuki obese diabetes) mouse	Non-obese C57Bl6 (Akita) mutant mouse ALS (alloxan sensitive)/Lt mouse
<b>Diet/nutrition-induced models</b>	Israeli Sand rat (Psammomys obesus) Spiny mouse <b>C57Bl6J mouse</b>	
<b>Chemically-induced</b>	GTG (goldthioglucose) treated obese mouse	ALX or STZ adult models Neonatal STZ rat
<b>Surgically-induced</b>	VMH (ventromedial hypothalamus) lesioned dietary obese rat	Partial pancreatectomized animals
<b>Genetically modified animals</b>	$\beta_3$ receptor -/- mouse UCP1 (uncoupling protein) -/- mouse	Transgenic or -/- mice of genes implicated in insulin resistance (e.g. IRS-1, IRS-2, GLUT-4), lipid and glucose metabolism (e.g. PPARs) and insulin secretion (GLUT-2, Glukokinase, IGF-IR), human islet amyloid polypeptide (hIAPP) transgenic rodents

(Adapted from *Chatzigeorgiou, 2009*)

### 3.4 High-fat feeding as a model for the study of T2DM

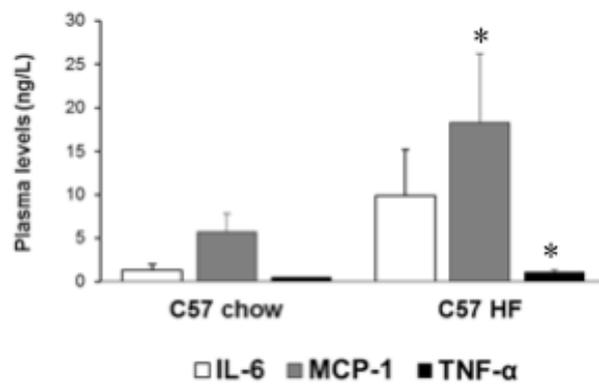
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In particular we have found ([Bernardi, submitted data](#)): (i) fat deposition; (ii) hyperglycemia, hyperinsulinemia and insulin resistance (**Figure 2**); (iii) low-grade inflammation (**Figure 3**). In conclusion, high-fat fed C57Bl6 mice develop a form of obesity-induced T2DM presumably caused by an accumulation of lipids leading to dysfunction of pancreatic islets and peripheral organs as well as adipose tissue enlargement and low-grade inflammation.



**Figure 2. Glucose and insulin in high-fat diet fed mice**

Blood glucose (mM) at 0, 15, 16 and 120 min during an IPGTT performed after 6 weeks (A) and 12 weeks (B) of HFD; Serum insulin (mM) at 0, 15, 16 and 120 min during an IPGTT performed after 6 weeks (C) and 12 weeks (D) of HFD; Blood glucose (mM) at 0, 15, 16 and 120 min during an IPITT performed after 6 weeks (E) and 12 weeks (F) of HFD. Data are reported as mean  $\pm$  SEM; \*  $p < 0.05$  vs C57 chow. Abbreviations: chow, chow fed; HF, high-fat diet fed; HFD, high-fat diet; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test (Bernardi, submitted data).



**Figure 3. High-fat diet-induced pro-inflammatory changes**

Circulating levels of IL-6; MCP-1; and TNF-  $\alpha$  in C57 chow (n=9) and C57 HF (n=9) mice. Data is expressed as mean  $\pm$  SEM. \* p < 0.05 vs C57 chow; † p < 0.005 vs C57 chow. Chow, chow fed; HF, high-fat diet fed; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . (Bernardi, submitted data)

# EXPERIMENTAL STUDY

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## Introduction and aim of the study

TRAIL is a member of the TNF superfamily of proteins, expressed either as a type II membrane or as a soluble protein [Di Pietro, 2004]. Among the putative physiological activities of TRAIL, the best-characterized function is the induction of apoptosis in tumor, infected or transformed cells, while leaving most non-transformed cells unharmed. TRAIL and its receptors have initially been implicated in immune surveillance, in particular against hematological malignancies [Secchiero, 2008]. Besides having been extensively studied as anticancer agent, TRAIL has also shown some therapeutic potential as anti-atherosclerotic and anti-diabetogenic molecule. In particular, in recent experimental works TRAIL was able to reduce the accelerated atherosclerosis observed in streptozotocin (STZ)-treated ApoE<sup>-/-</sup> mice [Secchiero, 2006] and to ameliorate the natural history of T1DM, due to its pro-apoptotic properties against activated macrophages and T cells [Zauli, 2010].

The first experimental evidences demonstrating TRAIL effects on T1DM were presented by Lamhamedi-Cherradi and colleagues [Sedger, 2002; Mi, 2003; Lamhamedi-Cherradi, 2003], proving that blockade of TRAIL signalling in NOD mice, or diabetes induction by STZ in TRAIL<sup>-/-</sup> mice, significantly worsened the natural history of the disease. Consistent with these earlier observations, our group has shown that TRAIL treatment reduces significantly glucose levels in rats with STZ-induced T1DM [Zauli, 2010]. This could be explained with the potent immune-regulatory effect of TRAIL, which therefore could be protective towards T1DM.

Whether TRAIL could ameliorate T2DM has only partially been answered. T2DM has been suggested to be caused by a breakdown in lipids dynamics, often reflected by elevated levels of triglycerides and circulating free FFA, which would ultimately lead to an excessive deposition of fat in various non-adipose tissues causing insulin resistance and  $\beta$ -cells abnormalities. High-fat diet (HFD) is given to reproduce obesity-induced insulin resistance, and T2DM [Turner, 2007]. Di Bartolo and colleagues have found that TRAIL<sup>-/-</sup> ApoE<sup>-/-</sup> mice on a HFD gained more body weight and that they displayed higher fasting glucose and pro-inflammatory cytokines levels than ApoE<sup>-/-</sup> mice [Di Bartolo, 2011]. So, we hypothesized that TRAIL delivery could reduce body weight, lower fasting glucose and pro-inflammatory cytokines levels, overall ameliorating T2DM. On this basis, the aim of our study was to

determine whether systemic TRAIL delivery would prevent the metabolic abnormalities due to a HFD in C57Bl6 mice [[Bernardi, 2012c](#)].

## Materials and methods

### 4.1 Animals and Experimental protocol

Twenty-seven wild-type male mice C57bl6 aged 8 weeks were randomly allocated to standard chow diet (C57 chow), HFD (C57 HF) or HFD + TRAIL (C57 HF + TRAIL) for 12 weeks. The animals were kept in a temperature-controlled room (22±1°C) on a 12- hours light/dark cycle with free access to food and water and they were fed ad libitum for the length of the study. The standard chow diet had 19.6% of protein, 4.6% of total fat, 4.5% of crude fibre providing a digestible energy of 14.3 MJ/Kg, the HFD had 22.6% of protein, 23.5% of total fat, 5.4% of crude fibre providing a digestible energy of 19 MJ/Kg. Whereas C57 chow and C57 HF mice were injected intraperitoneally (ip) 100 µl of saline, C57 HF + TRAIL mice were injected ip 10 mg of TRAIL in 100 µl of saline every week for the 12 weeks of the study. The drug was a recombinant (r) human (h) histidine 6-tagged hTRAIL (114-281), produced in bacteria and resuspended in buffered saline before the injection. Body composition and food intake were evaluated every four weeks in all the mice studied, then measurements by indirect calorimetry were performed at week eleven and IPGTT (intraperitoneal glucose tolerance test) and IPITT (intraperitoneal insulin tolerance test) were performed at week twelve. At the end of the study, the animals were then anesthetized by an ip injection of pentobarbitone at a dose of 100 mg/Kg body weight. Blood was collected from the left ventricle, centrifuged and plasma was stored at -20°C for analysis. Adipose tissue, liver, and skeletal muscle were kept for further analysis. Principles of laboratory animal care were followed as well as specific national laws where applicable. This study was approved by the animal ethic committee AMREP (*the number of approval was 0947 and the study period was May 2010 - May 2012*).

### 4.2 Recombinant human TRAIL production

Rh-histidine 6-tagged TRAIL (114-281) was produced in bacteria BL21, transformed with a pTrc-His6 TRAIL vector. The gene sequence (residues between 114 and 281) was preceded by a histidine 6-tag and by a promoter that was stimulated by bacterial logarithmic phase growth. Protein extraction was performed by sonication and the use of different buffers, while purification was obtained by affinity chromatography on Ni<sup>2+</sup> affinity resin. Purification consisted of three steps: binding proteins to the Ni<sup>2+</sup> affinity resin, washing off unbound

proteins, and eluting those purified. These purified preparations were then dialyzed for 48 hours in saline solution, aliquotated and stored at -80°C. The Chromogenic Limulus Amebocyte Lysate (LAL) Test (*BioWhittaker*) was used to quantify bacterial endotoxins, which resulted always lower than 0.1EU/ml. Then, the purity of each TRAIL preparation was evaluated by loading 2 µg of rh-TRAIL in 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), with or without β-mercaptoethanol. The gels were then stained with the gelcode Silver SNAP Staining kit (*Pierce, Boston, MA*). Finally, TRAIL biological activity was determined in vitro by propidium iodide staining and flow cytometric analysis of apoptosis in the TRAIL-sensitive HL-60 cell line. Rh- his6-TRAIL will be indicated as TRAIL throughout the text.

#### 4.3 Determination of body composition

Total body mass, fat mass, and lean mass were evaluated at baseline, and every four weeks by EchoMRI (*Echo Medical Systems, Houston Texas*).

#### 4.4 Food intake and energy intake

Food intake was measured every four weeks placing in the cages pellets previously weighed in total. The food that was left over was then collected and weighed to find the amount eaten. Energy intake was measured according to the digestible energy provided by both diets.

#### 4.5 Glucose, insulin, lipids, TRAIL and pro-inflammatory cytokines measurements

Glucose was measured using a glucometer (*AccuCheck II; Roche, NSW, Australia*) during the IPGTT, IPITT and at the end of the study, at fasting. Insulin was measured by ELISA (*Millipore, Cat# EZRMI-13K*) during the IPGTT and at the end of the study, at fasting. The IPGTT was performed on day 1 after the last injection of TRAIL or saline. 2g/kg of glucose were injected ip after an overnight fast and bloods were collected at baseline, 15', 60' and 120' after the injection. The IPITT was performed on day 4 after the last injection of TRAIL or saline. 1U/kg of insulin was injected ip after a 6 hours fast and bloods were collected at baseline, 15', 60', and 120' after the injection. At the end of the study, at fasting, we also measured: total

cholesterol, LDL, HDL and triglycerides by COBAS INTEGRA 200, FFAs by a colorimetric kit (*Wako Pure Chemical Industries, Osaka, Japan*) adiponectin and interleukin-6 (IL-6), interleukin-10 (IL-10), leptin, plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) by Luminex with a commercial Multiplex kit (*Millipore, Cat#MADPK-71K and #MADPK-71K-ADPN*). Circulating TRAIL was determined by ELISA (*R&D Systems, Minneapolis, MN*), as previously described [[Campioni, 2005](#)]. Sensitivity of the TRAIL assay was 2.9 pg/ml and the intra- and inter- assay coefficients of variation (CV) were 3.9% and 6%, respectively.

#### 4.6 Indirect calorimetry studies

O<sub>2</sub> consumption rate (V<sub>O2</sub>) and CO<sub>2</sub> production rate (V<sub>CO2</sub>) were determined before the last injection of TRAIL by indirect calorimetry (*Oxymax, Columbus Instruments*), with 1 mouse per chamber. Each chamber was equipped with a 3-dimensional (xyz) infrared beam system (OPTO- M3) to record locomotor activity. Conditioned fresh air at 21±0.5°C and 55%±5% relative humidity was pumped into the chambers at 0.6 l/min. The animals were acclimated to the chambers for 1 day, and V<sub>O2</sub> and V<sub>CO2</sub> measurements taken every 30 minutes were collected and recorded on a computer over the next 24 hours. During the 12-hours dark/12-hours light phases, mice had free access to food and water. The energy expenditure was expressed as V<sub>O2</sub>. RER was calculated as V<sub>CO2</sub> production/V<sub>O2</sub> consumption, with the values of 1 or 0.7 indicating 100% CHO or 100% fat oxidation, respectively.

#### 4.7 Palmitate oxidation rate

At the end of the study, to evaluate FFA oxidation rate in the skeletal muscle of the mice studied, palmitate oxidation rate was measured in skeletal muscle homogenates using a modified method described by Turner and associates [[Turner, 2007](#)]. Muscles were homogenized in 19 volumes of ice-cold 250 mmol/l sucrose, 10 mmol/l Tris-HCl, and 1 mmol/l EDTA, pH 7.4. For assessment of substrate oxidation, 50  $\mu$ l of muscle homogenate was incubated with 450  $\mu$ l reaction mixture (pH 7.4). Final concentrations of the reaction mixture were (in mmol/l): 100 sucrose, 80 KCl, 10 Tris- HCl, 5KH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 2 malate, 2 ATP, 1 DTT, 0.2 EDTA and 0.3% FFA free BSA. Substrates were 0.2 mmol/l [<sup>1-14</sup>C]palmitate (0.5  $\mu$ Ci) plus 2 mmol/l carnitine and 0.05 mmol/l coenzyme A. After 90 min of incubation at 30°C, the reaction

was stopped by the addition of 100  $\mu$ L of ice-cold 1 mol/l perchloric acid. CO<sub>2</sub> produced during the incubation was collected in 100  $\mu$ l of 1 mol/l sodium hydroxide. <sup>14</sup>C counts present in the acid-soluble fraction were also measured and combined with the CO<sub>2</sub> values to give the total palmitate oxidation rate.

#### 4.8 Gene expression quantification by Real-time PCR

Three micrograms of total RNA extracted from adipose tissue, liver, and skeletal muscle were used to synthesize cDNA with Superscript First Strand synthesis system for RT-PCR (*Gibco BRL*). Bcl-2, caspase3, IL-6, MCP-1, and TNF- $\alpha$  gene expression were analysed by real-time quantitative RT-PCR using the TaqMan system based on real-time detection of accumulated fluorescence. Fluorescence for each cycle was quantitatively analysed by an ABI Prism 7700 Sequence Detection System (*Perkin-Elmer Inc*). Gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18s ribosomal RNA. Primers and Taqman probes were constructed with the help of Primer Express (*ABI Prism 7700, Perkin-Elmer Inc*).

#### 4.9 In vivo apoptosis

Adipose tissue apoptosis was detected by Transferase-mediated dUTP Nick End Labeling (TUNEL) staining. Apoptosis was identified by 3' in situ end labeling of fragmented DNA with Terminal deoxynucleotidyltransferase (TdT). After fixation and permeabilisation with 0.1% Triton X-100 and 0.1% sodium citrate fresh solution, 20  $\mu$ m frozen sections of adipose tissue were incubated with TUNEL reaction mixture, according to the manufacturer's instructions (*Roche diagnostic, Indianapolis, IN*) and mounted with DAPI to be seen under fluorescence. The number of (TUNEL)-positive cells was calculated and expressed as percentage of TUNEL-positive cells per frame.

#### 4.10 Statistical analysis

Data were calculated and shown as means  $\pm$  standard errors of the mean. Comparisons between STZ-treated animals and normal controls were performed with Student t test and with

$\chi^2$  test. Differences in parameters mean values across study phases were analyzed using ANOVA for repeated measures. Statistical significance was defined as  $p < 0.05$ .

## Results

### 5.1. TRAIL significantly reduces the increased adiposity associated with a high fat diet in spite of promoting a higher energy intake

C57 HF mice became obese (**Figure 4A**) and displayed increased adiposity after 4, 8 and 12 weeks compared to C57 chow mice (**Figure 4B**). The increase in adiposity due to a HFD was significantly delayed by TRAIL treatment. After 4 weeks of study, C57 HF + TRAIL mice did not differ from C57 chow mice in terms of adiposity, whereas their fat mass was significantly lower than the fat mass of C57 HF untreated. After 8 and 12 weeks of study TRAIL significantly reduced the adiposity of the mice high fat fed (**Figure 4B**). There was no significant difference in terms of lean mass between the groups studied (**Figure 4C**). C57 HF mice ate significantly less ( $p < 0.05$ ) than the other mice groups during the length of the study. Given the composition of both diets, C57 HF+TRAIL mice displayed the highest energy intake among the groups, eating  $0.094 \pm 0.006$  mj/day, compared to C57 chow mice, eating  $0.057 \pm 0.004$  mj/day, and C57 HF, eating  $0.06 \pm 0.004$  mj/day ( $p < 0.05$  vs both C57 chow and C57 HF+TRAIL). It is noteworthy that repeated ip injections of TRAIL were safe, since mice treated with TRAIL didn't show gross abnormalities at necroscopic examination with respect to the mock treated mice. Moreover, human TRAIL was detectable by ELISA in sera of the injected mice up to 4 days after ip injections (data not shown).

### 5.2. TRAIL significantly reduces hyperglycaemia and hyperinsulinemia during an IPGTT as well as hyperinsulinemia at fasting and improves the peripheral response to insulin in high fat fed mice

The IPGTT (**Figure 5A**) showed that the HFD resulted in a significant impairment of glucose clearance, leading to hyperglycemia at all the time-points examined, which was significantly reduced by TRAIL at all the time-points (**Figure 5A**). The area under the curve was  $1291.08 \pm 240.58$  in C57 chow mice,  $2744.25 \pm 152.72$  in C57 HF ( $p < 0.05$  vs C57 chow) and  $2277.9 \pm 186.13$  in C57 HF+TRAIL ( $p < 0,01$  vs C57 HF). As for insulin levels during the same

IPGTT (**Figure 5B**), C57 HF mice displayed a significant hyperinsulinemia compared to C57 chow mice, which was significantly reduced by TRAIL treatment (**Figure 5B**). The area under the curve was  $152.44 \pm 1.87$  in C57 chow mice,  $278.15 \pm 24.52$  in C57 HF ( $p < 0.05$  vs C57 chow) and  $160.50 \pm 17.13$  in C57 HF+TRAIL ( $p < 0.01$  vs C57 HF). The IPITT showed that TRAIL treatment improved the peripheral response to insulin in mice fed a HFD, lowering their glucose levels (C57 HF+TRAIL vs C57+HF) at all time-points examined and reaching the significance at 60 and 120 minutes after the insulin challenge (**Figure 5C**). At the end of the study, although glucose levels were not significantly lower in C57 HF+TRAIL mice compared to the mice untreated, C57 HF+TRAIL mice presented lower levels of insulin at fasting than C57 HF mice (**Figure 5D**). Serum total cholesterol, HDL and LDL increased after HFD without being affected by TRAIL treatment ( $p < 0.001$  vs C57 chow). Nor serum tryglicerides nor free fatty acid change among the groups being  $0.59 \pm 0.09$  mmol/L in C57 chow mice;  $0.61 \pm 0.19$  mmol/L in C57 HF mice and  $0.58 \pm 0.225$  mmol/L in C57 HF TRAIL.

### 5.3. TRAIL treatment significantly reverses the changes in substrate utilization induced by HFD and ameliorates *ex vivo* palmitate oxidation

At the end of the study, indirect calorimetry showed that the VO<sub>2</sub> increased significantly after a HFD (**Table 5**). Heat production increased in both C57 HF and C57 HF+TRAIL mice compared to the controls, whereas locomotor activity was significantly reduced in the C57 HF mice. Interestingly, the measurement of the respiratory exchange ratio (RER), which is the ratio between VCO<sub>2</sub> produced and VO<sub>2</sub> burnt, showed that followed a HFD significantly shifts the metabolism towards a nearly complete fat consumption as fuel, as the C57 HF mice displayed a RER significantly lower than the RER of the controls (**Table 5**). TRAIL significantly reversed the reduction of the RER that follows a HFD as the C57 HF+TRAIL mice displayed an RER significantly higher than the RER of the C57 HF mice (**Table 5**). In parallel experiments, we found that *ex vivo* palmitate oxidation (nmol/min/g) in skeletal muscle, measured after 12 weeks of HFD, significantly increased in C57 HF vs C57 chow ( $16.56 \pm 1.1$  vs  $14.3 \pm 1.1$ , respectively,  $p < 0.05$ ). However, TRAIL treatment further increased it compared to C57 HF ( $19.53 \pm 1.8$  vs  $16.56 \pm 1.1$ , respectively,  $p < 0.05$ ).

**Table 5. Summary of indirect calorimetry and muscular palmitate oxidation studies**

Parameter	C57 chow	C57 HF	C57 HF + TRAIL
<b>VO<sub>2</sub> (ml/g/h)</b>	5.04 ± 0.31	6.40 ± 0.23 *	6.6 ± 0.42 *
<b>Heat (Kcal/HR)</b>	0.56 ± 0.01	0.72 ± 0.03 *	0.81 ± 0.03 *
<b>Locomotor activity</b>	21074 ± 1365	15077 ± 861 *	24468 ± 2055 #
<b>RER (VCO<sub>2</sub>/VO<sub>2</sub>)</b>	0.89 ± 0.01	0.77 ± 0.007 *	0.82 ± 0.0009 *#
<b>Palmitate oxidation (nmol/min/g)</b>	14.4 ± 1.1	16.56 ± 1.1	19.53 ± 1.8

All the data are expressed as mean ± SEM; \* p < 0.05 vs C57 chow; # p < 0.05 vs C57 HF. C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet + TRAIL (C57 HF + TRAIL); High fat (HF); Respiratory exchange ratio (RER); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (Adapted from [Bernardi, 2012c](#))

#### 5.4. TRAIL significantly lowers circulating levels and gene expression of pro-inflammatory cytokines

As for the circulating levels of the adipokines measured, adiponectin decreased significantly in the mice high fat fed with respect to the controls but it did not change significantly after TRAIL treatment. Adiponectin levels were respectively 11 ± 1 µg/mL in C57 chow, 7 ± 1 µg/mL in C57 HF, 9 ± 1 µg/mL in C57 HF + TRAIL mice. Leptin levels also did not change between the C57 HF and C57 HF + TRAIL mice, being 1350 ± 248 pg/mL in C57 chow, 12959 ± 1381 pg/mL in C57 HF and 10909 ± 1418 pg/mL in C57 HF+TRAIL mice.

Among the cytokines measured, IL-6 increased significantly in C57 HF mice and was significantly reduced by TRAIL (**Figure 6A**). IL-10 did not change between the groups studied, being 26 ± 14 pg/mL in C57 chow, 37 ± 10 pg/mL in C57 HF and 20 ± 12 pg/mL in C57 HF + TRAIL mice. MCP-1 followed the same trend of IL-6, being 570 ± 328 pg/mL in C57 chow mice, 1750 ± 1559 pg/mL in C57 HF mice and 300 ± 222 pg/mL in C57 HF+TRAIL mice. PAI-1 was 1326 ± 264 pg/mL in C57 chow; 1593 ± 198 pg/mL in C57 HF and 1332 ± 214 pg/mL in C57 HF+TRAIL mice. TNF-α levels followed the same trend of IL-6 (**Figure 6B**). This was consistent

with the significant reduction of hepatic IL-6 gene expression as well as with the significant downregulation of TNF- $\alpha$  in adipose tissue and skeletal muscle, which were observed after TRAIL treatment (**Figure 7**). A significant downregulation of MCP-1 in adipose tissue was also observed (**Table 6**)

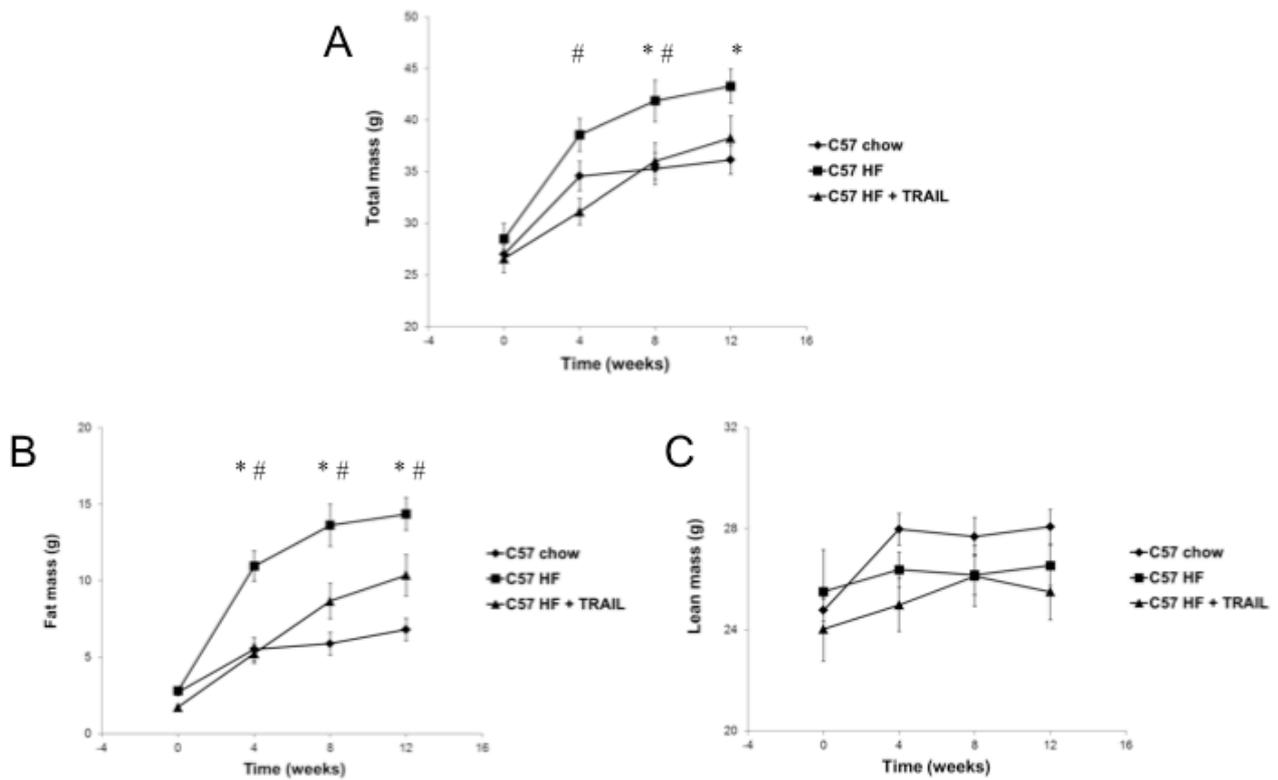
### 5.5. TRAIL treatment modulates adipose tissue gene expression and promotes adipose tissue apoptosis

Apart from IL-6 and TNF- $\alpha$ , the expression of genes related to apoptosis, such as Bcl-2 and caspase3 was analyzed. As summarized in **Table 6**, TRAIL treatment was associated with a significant up-regulation of caspase-3. Consistent with this induction, TRAIL treated C57 HF mice displayed a significant increase (average 2.8 fold;  $p < 0.001$ ) of the percentage of apoptotic cells in adipose tissue sections.

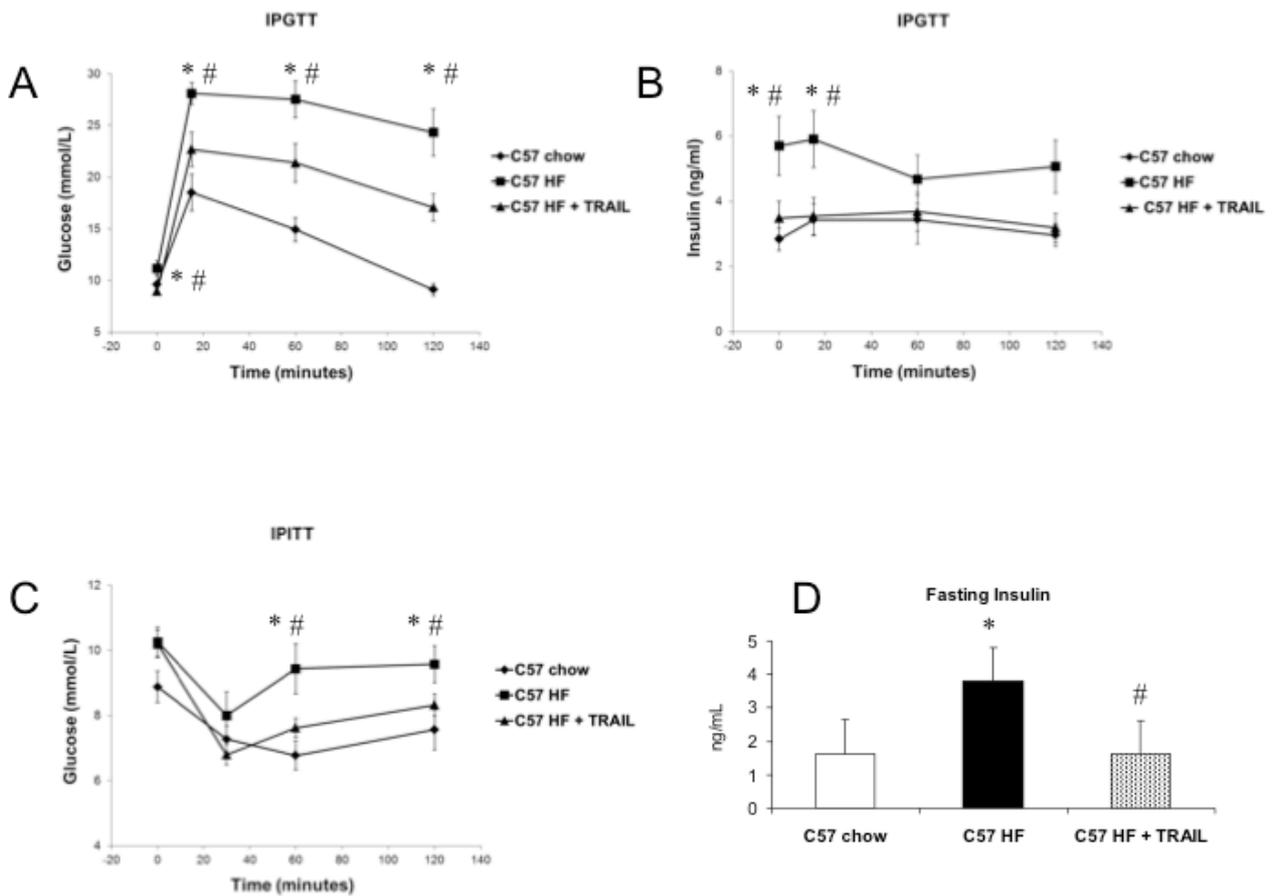
**Table 6. Adipose tissue gene expression**

Gene	C57 chow	C57 HF	C57 HF + trail
<b>Bcl-2</b>	1 $\pm$ 0.29	5.80 $\pm$ 1.58 *	4.21 $\pm$ 0.47 *
<b>Caspase-3</b>	1 $\pm$ 0.25	1.96 $\pm$ 0.32 *	3.91 $\pm$ 0.60 *#
<b>IL-6</b>	1 $\pm$ 0.13	2.15 $\pm$ 0.54 *	1.69 $\pm$ 0.44
<b>MCP-1</b>	1 $\pm$ 0.16	5.26 $\pm$ 1.34	2.4 $\pm$ 0.42 *#
<b>PPAR<math>\gamma</math></b>	1 $\pm$ 0.12	5.93 $\pm$ 2.12 *	2.04 $\pm$ 0.17 *
<b>TNF-<math>\alpha</math></b>	1 $\pm$ 0.28	3.82 $\pm$ 0.91 *	1.02 $\pm$ 0.20 #

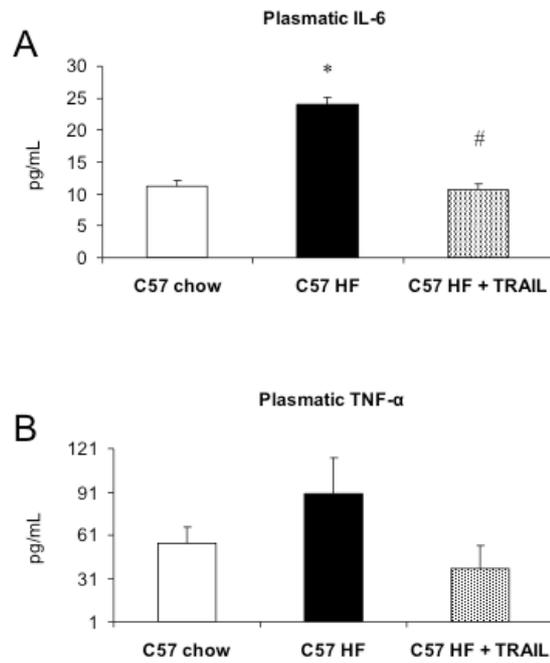
Adipose tissue mRNA expression of Bcl-2, BMP-7, Caspase-3, IL-6, PPAR $\gamma$ , TNF- $\alpha$  in C57 chow, C57 HF, C57 HF + TRAIL mice. mRNA expression is reported as relative gene units; data is expressed as mean  $\pm$  SEM. \*  $p < 0.05$  vs C57 chow; #  $p < 0.05$  vs C57 HF. B-cell lymphoma 2 (BCL2); Bone morphogenetic protein 7 (BMP7); C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet + TRAIL (C57 HF + TRAIL); High fat (HF); Interleukin-6 (IL-6); Monocyte chemoattractant protein-1 (MCP-1); Peroxisome proliferator activated receptor (PPAR $\gamma$ ); Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (Adapted from [Bernardi, 2012c](#))



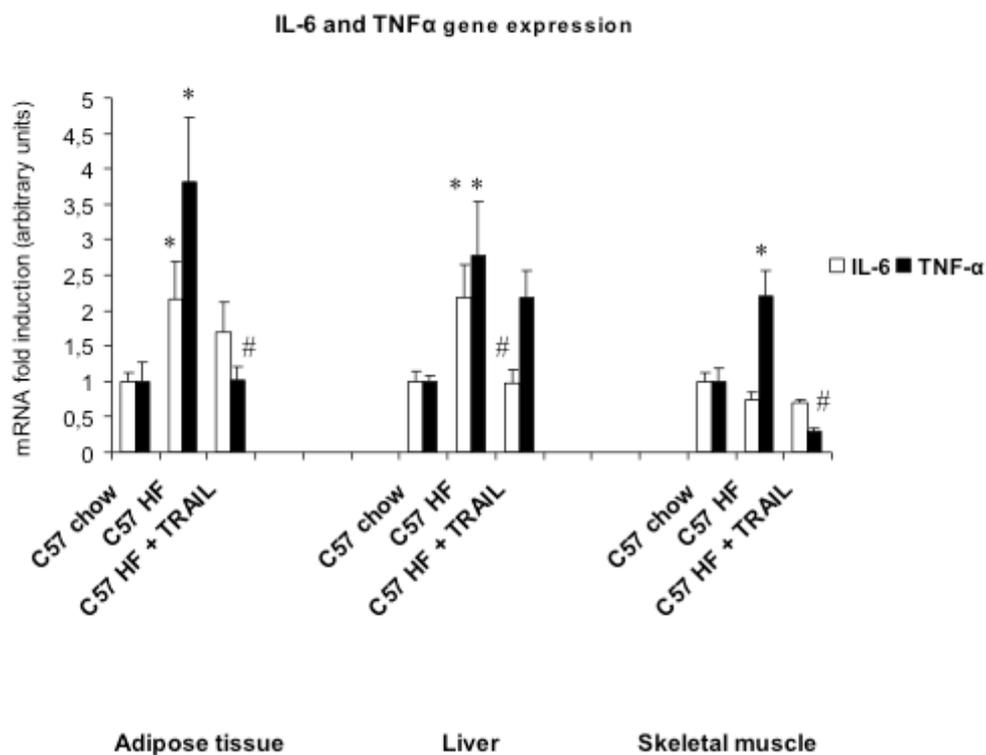
**Figure 4. *Body mass composition*** (A) Total mass (g) at baseline, 4, 8 and 12 weeks of study; (B) Fat mass (g) at baseline, 4, 8 and 12 weeks of study; (C) Lean mass (g) at baseline, 4, 8 and 12 weeks of study. Data is expressed as means±SEM. \*  $p < 0.05$  C57 HF vs C57 chow; #  $p < 0.05$  C57 HF vs C57 HF+TRAIL. C57bl6 mice allocated to standard chow diet (C57 chow); C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet+TRAIL (C57 HF+TRAIL); High fat (HF); Tumor necrosis factor -related apoptosis-inducing ligand (TRAIL).



**Figure 5. *Glucose and insulin levels.*** (A) Blood glucose (mmol/L) during an IPGTT at 0', 15', 60', 120'; (B) Serum insulin (ng/mL) during an IPGTT at 0', 15', 60', 120'; (C) Blood glucose (mmol/L) during an IPITT at 0', 30', 60', 120'; (D) Plasmatic insulin (ng/mL) at fasting. Data is expressed as mean  $\pm$  SEM; \*  $p < 0.05$  C57 HF vs C57 chow; #  $p < 0.05$  C57 HF vs C57 HF+TRAIL. C57bl6 mice allocated to standard chow diet (C57 chow); C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet + TRAIL (C57 HF + TRAIL); Intraperitoneal glucose tolerance test (IPGTT); Intraperitoneal insulin tolerance test (IPITT); High fat (HF); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).



**Figure 6. *Circulating levels of pro-inflammatory cytokines.*** Plasmatic levels of IL-6 and TNF- $\alpha$  in C57 chow, C57 HF and C57 HF+TRAIL mice. IL-6 and TNF- $\alpha$  are measured as pg/mL. Data is expressed as means $\pm$ SEM; \* $p$ <0.05 C57 HF vs C57 chow; #  $p$ <0.05 C57 HF vs C57 HF+TRAIL. C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet+TRAIL (C57 HF + TRAIL); high fat (HF); Interleukin-6 (IL-6); Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).



**Figure 7. Gene expression of pro-inflammatory cytokines.** (A) Adipose, hepatic and muscular mRNA expression of IL-6 and TNF- $\alpha$  in C57 chow, C57 HF and C57 HF + TRAIL mice. mRNA expression is reported as relative gene units; data is expressed as mean  $\pm$  SEM. \*  $p < 0.05$  C57 HF vs C57 chow; #  $p < 0.05$  C57 HF + TRAIL vs C57 HF. C57bl6 mice allocated to high fat diet (C57 HF); C57bl6 mice allocated to high fat diet + TRAIL (C57 HF + TRAIL); high fat (HF); tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); Interleukin-6 (IL-6); Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).

## Discussion

It has been demonstrated that HFD-fed C57Bl6 mice develop a significant impairment in glucose tolerance and a 40% reduction in insulin stimulated glucose uptake in skeletal muscle [Turner, 2007]. This relies on the notion that an oversupply of lipids, leading to an abnormal accumulation of fat in adipose and non-adipose tissues such as muscle and liver, plays an important role in the etiology of insulin resistance and later on in the demise of the  $\beta$ -cell in T2DM [McGarry, 2002]. TRAIL deficiency has recently been found to promote numerous features of T2DM in mice high fat fed. In our work, whose aim was the study of the effects of TRAIL delivery in mice high fat fed, we have documented the ability of recombinant TRAIL to significantly reverse the metabolic abnormalities due to an oversupply of lipids and thus to slow down the natural history of T2DM. TRAIL treatment reduced significantly the hyperglycemia and the hyperinsulinemia displayed by high fat fed mice during an IPGTT. Consistent with this, TRAIL treatment ameliorated their peripheral response to insulin, lowering significantly glucose levels during an IPITT.

An increased utilization of free fatty acid for fuel in skeletal muscle is a typical feature of HFD-related insulin resistance [Turner, 2007]. This phenomenon has been explained either as a compensatory response to a greater availability of fatty acids substrates or as the consequence of an abnormal peripheral response to insulin, so that the body cannot burn glucose efficiently. As expected, in our study, C57 HF mice shifted their metabolism from glucose towards lipids consumption, according to their lower RER and increased skeletal muscle palmitate oxidation rate. TRAIL treatment was associated with an improvement of the peripheral response to insulin and an increased RER. Quite surprisingly though, skeletal muscle palmitate oxidation rate was significantly higher in C57 HF+TRAIL mice compared to those untreated. To clarify this data two aspects should be taken into account. Firstly, any muscular lipid overload may be linked to a reduction in lean mass, as it is seen in insulin resistance, which in turn would lead to low rates of palmitate (or FFAs) oxidation. Secondly, the *ex vivo* assessment of FFA oxidation is performed under favourable conditions of substrate availability in an environment free of regulatory factors that might affect this process [Turner, 2007]. However, since mitochondrial dysfunction has been pointed out as one of the earliest defects that predispose to lipid accumulation and insulin resistance, the increased palmitate oxidation

rate observed in high fat fed mice treated with TRAIL is consistent with a protective effect of TRAIL against mitochondrial dysfunction and therefore lipid accumulation and insulin resistance.

The reversal of the metabolic abnormalities due to a HFD upon TRAIL treatment was accompanied by the effect of TRAIL on mice adiposity. TRAIL treatment significantly reduced the deposition of fat mass induced by the HFD during the length of the study. This is consistent with what has been reported by Di Bartolo and colleagues [*Di Bartolo, 2011*], who found that mice lacking TRAIL presented an increased deposition of fat. A different group has recently reported an association between serum TRAIL concentrations, serum lipid levels and body adiposity in healthy adults [*Choi, 2004*]. In this cohort, TRAIL serum concentrations were shown to increase proportionally with the body mass and circulating lipids levels. So it is conceivable, that TRAIL, which has been shown to counteract fat accumulation, increases as the body mass increases because of a compensatory mechanism set up by the body to respond to fat gain. In our work, the significant reduction in the adiposity gained after a HFD that followed TRAIL delivery was not due to a reduction in food intake or to a change in circulating adiponectin and leptin. On the contrary, our data shows that C57 HF+TRAIL mice ate as much as C57 chow mice, whereas C57 HF mice displayed a reduction in their food intake, possibly related to the increased adiposity which would have lead them to move less for seeking food. Since the HFD is slightly hypercaloric compared to the standard diet, C57 HF+TRAIL mice displayed the highest caloric intake during the length of the study.

The analyses performed on the fat of the mice suggest an effect of TRAIL on fat apoptosis and differentiation. TRAIL best known function is indeed the induction of apoptosis, which occurs through the activation of the extrinsic apoptotic pathway and a cross-talk between extrinsic and intrinsic pathways, triggered by the binding of a soluble TRAIL trimer to its receptors TRAIL-R1 and TRAIL-R2 [*Bernardi, 2012a*]. This effect is selective, so whereas activated or transformed cells are sensitive to TRAIL-induced apoptosis, non-transformed cells are generally not affected by it [*Bernardi, 2012a*]. However, a recent work has shed light on the ability of TRAIL to induce apoptosis of human pre-adipocytes [*Mader, 2012*]. In this work, apoptosis was caused by a synergistic interaction between resveratrol and TRAIL, which activated the intrinsic apoptosis pathway, and caspases. Here, we found an up- regulation of caspase-3 as well as an increase of apoptotic nuclei within the adipose tissue of mice treated with TRAIL. So, a possible mechanism underlying the reduction of fat mass by TRAIL treatment

could be a pro-apoptotic effect against pre-adipocytes. However, there may be other mechanisms underlying our observations, such as an effect of TRAIL on adipocyte differentiation, which has recently been reported by Keuper and colleagues [[Keuper, 2011](#)]. Consistent with this, in our study, TRAIL treatment significantly downregulated PPAR $\gamma$ , a well known regulator of adipogenesis and neutral lipid storage, which induces fat cell differentiation [[Lehrke, 2005](#)].

The improvement of T2DM metabolic abnormalities following TRAIL treatment could be explained with its effect on adiposity, which might then have influenced pro-inflammatory cytokines levels and glucose metabolism. It is in fact well known that obesity is an independent predictor of insulin resistance and T2DM [[Collins, 2011](#)]. Clinical studies as the Finnish Diabetes Prevention Study [[Tuomilhto, 2001](#)] have shown that T2DM can be prevented by lifestyle modifications leading to weight loss. Among the multiple mechanisms linking obesity to diabetes, inflammation is a common feature that has been implicated in the pathophysiology of both diseases. Several evidences have in fact proven the existence of an association between obesity, low-grade inflammation and metabolic disturbances such as insulin resistance and T2DM [[Xu, 2003](#)]. For instance, weight loss was able to reduce inflammatory markers [[Esposito, 2003](#); [Bastard, 2000](#)] with an improvement of insulin sensitivity [[Ryan, 1994](#)], whereas the deficiency of pro-inflammatory cytokines, such as TNF- $\alpha$ , was protective from obesity-induced insulin resistance [[Hotamisligil, 1994](#)]. In addition, IL-6, MCP-1 and TNF- $\alpha$  seem to correlate to body weight and they have all been shown to link obesity to insulin resistance [[Di Gregorio, 2005](#)]. So, TRAIL treatment reduced significantly the fat mass of the mice by inducing apoptosis and/or affecting adipocytes differentiation. This might have in turn led to a significant reduction of circulating and hepatic IL-6, as well as to a significant downregulation of MCP-1 and TNF- $\alpha$  in the adipose tissue and TNF- $\alpha$  in the skeletal muscle, possibly explaining its positive metabolic effects. However, we cannot exclude a direct anti-inflammatory effect of TRAIL, which could have contributed to the amelioration of T2DM metabolic disturbances, as previous works have clearly demonstrated that TRAIL induces the apoptosis of activated macrophages and T cells in different tissues. Further studies are needed to clarify this issue.

In conclusion, consistent with what has been previously reported, this work sheds light on the possible anti-adipogenic and anti-inflammatory effects of TRAIL and opens new

therapeutic possibilities [[Thomas, 2012](#)] against weight gain, systemic inflammation, insulin resistance and T2DM.

## LIST of PUBLICATIONS (2010-2013)

### Patents

Merlin Thomas, Giorgio Zauli, Paola Secchiero, Bruno Fabris, **Stella Bernardi**. Apoptosis-inducing molecules and uses therefor. 2012/9/8 WO 2012117336

### Original articles

1. Catena C, **Bernardi S**, Sabato N, Grillo A, Ermani M, Sechi LA, Fabris B, Carretta R, Fallo F. Ambulatory arterial stiffness indices and non-alcoholic fatty liver disease in essential hypertension. *Nutr Metab Cardiovasc Dis.* 2013; 23(4):389-93
2. Tisato V, Toffoli B, Monasta L, **Bernardi S**, Candido R, Secchiero P, Zauli G. Patients affected by metabolic syndrome show decreased levels of circulating platelet derived growth factor (PDGF)-BB. *Clin Nutr.* 2013; 32(2):259-64
3. **Bernardi S**, Norcio A, Toffoli B, Zauli G, Secchiero P. Potential role of TRAIL in the management of autoimmune diabetes mellitus. *Curr Pharm Des.* 2012; 18(35): 5759-65
4. **Bernardi S**, Milani D, Fabris B, Secchiero P, Zauli G. TRAIL as biomarker and potential therapeutic tool for cardiovascular diseases. *Curr Drug Targets.* 2012; 13(9): 1215-21
5. **Bernardi S**, Zauli G, Tikellis C, Candido R, Fabris B, Secchiero P, Cooper ME, Thomas MC. TNF-related apoptosis-inducing ligand significantly attenuates metabolic abnormalities in high fat fed mice reducing adiposity and systemic inflammation. *Clin Sci (Lond).* 2012; 123(9):547-55
6. **Bernardi S**, Burns WC, Toffoli B, Pickering R, Sakoda M, Tsorotes D, Grixti E, Velkoska E, Burrell LM, Johnston C, Thomas MC, Fabris B, Tikellis C. Angiotensin-converting enzyme 2 regulates renal atrial natriuretic peptide through angiotensin-(1-7). *Clin Sci (Lond)* 2012; 123(1): 29-37
7. **Bernardi S**, Secchiero P, Zauli G. State of art and recent developments of anti-cancer strategies based on TRAIL. *Recent Pat Anticancer Drug Discov.* 2012; 7(2): 207-17
8. **Bernardi S**, Zennaro C, Palmisano S, Velkoska E, Sabato N, Toffoli B, Giacomel G, Buri L, Zanconati F, Bellini G, Burrell LM, De Manzini N, Fabris B. Characterization and significance of ACE2 and Mas receptor in human colon adenoca. *J Renin Angiotensin Aldosterone Syst.* 2012; 13(1): 202-9
9. **Bernardi S**, Toffoli B, Zennaro C, Tikellis C, Monticone S, Losurdo P, Bellini G, Thomas MC, Fallo F, Veglio F, Johnston CI, Fabris B. High-salt diet increases glomerular ACE/ACE2 ratio leading to oxidative stress and kidney damage. *Nephrol Dial Transplant.* 2012; 27(5): 1793-800
10. Toffoli B, Pickering RJ, Tsorotes D, Bo W, **Bernardi S**, Kantharidis P, Fabris B, Zauli G, Secchiero P, Thomas MC. Osteoprotegerin promotes vascular fibrosis via a TGF- $\beta$ 1 autocrine loop. *Atherosclerosis.* 2011; 218(1): 61-68
11. **Bernardi S**, Severini GM, Zauli G, Secchiero P. Cell-based therapies for diabetic complications. *Experimental Diabetes Research.* 2012:872504
12. **Bernardi S**, Grimaldi F, Finato N, De Marchi S, Proclemer A, Sabato N, Bertolotto M, Fabris B. A pheochromocytoma with high ACTH and a silent lung nodule. *The American Journal of American Sciences.* 2011; 342(5): 429-432
13. Fischetti F, Candido R, Toffoli B, Durigutto P, **Bernardi S**, Carretta R, Tedesco F, Fabris B. Innate immunity, through late complement components activation, contributes to the development of early vascular inflammation and morphologic alterations in experimental diabetes. *Atherosclerosis.* 2011; 216:83-89
14. Toffoli B, **Bernardi S**, Candido R, Zacchigna S, Fabris B, Secchiero P. TRAIL shows potential cardioprotective activity. *Invest New Drugs.* 2012; 30(3): 1257-60
15. Fabris B, Candido R, Bortoletto M, Toffoli B, **Bernardi S**, Stebel M, Bardelli M, Zentilin L, Giacca M, Carretta R. Stimulation of cardiac apoptosis in ovariectomized hypertensive rats: potential role of the renin-angiotensin system. *J Hypertens.* 2011; 29:273-281
16. Tikellis C, **Bernardi S**, Burns WC. Angiotensin-converting enzyme 2 is a key modulator of the renin-angiotensin system in cardiovascular and renal disease. *Curr Opin Nephrol Hypertens.* 2011 20:62-68
17. Toffoli B, **Bernardi S**, Candido R, Sabato N, Carretta R, Corallini F, Secchiero P, Zauli G, Fabris B. Osteoprotegerin induces morphological and functional alterations in mouse pancreatic islets. *Mol Cell Endocrinol.* 2011; 331(1):136-42
18. **Bernardi S**, Candido R, Toffoli B, Carretta R, Fabris B. Prevention of accelerated atherosclerosis by AT1 receptor blockade in experimental renal failure. *Nephrol Dial Transplant.* 2011; 26(3):832-8
19. Thomas MC, Pickering RJ, Tsorotes D, Koitka A, Sheehy K, **Bernardi S**, Toffoli B, Nguyen-Huu TP, Head GA, Fu Y, Chindusting J, Cooper ME, Tikellis C. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res.* 2010; 107(7):888-97
20. Candido R, Toffoli B, Corallini F, **Bernardi S**, Zella D, Voltan R, Grill V, Celeghini C, Fabris B. Human full-length osteoprotegerin induces the proliferation of rodent vascular smooth muscle cells both in vitro and in vivo. *J Vasc Res.* 2010; 47(3):252-61
21. Fallo F, Dalla Pozza A, Sonino N, Lupia M, Tona F, Federspil G, Ermani M, Catena C, Soardo G, Di Piazza L, **Bernardi S**, Bertolotto M, Pinamonti B, Fabris B, Sechi LA. Non-alcoholic fatty liver disease is associated with left ventricular diastolic dysfunction in essential hypertension. *Nutr Metab Cardiovasc Dis.* 2009;19(9):646-53

## References

1. Adams JM, Cory S, **2007**. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol.* 19: 488-96
2. Appelman LJ, Tzachanis D, Grader-Beck T, **2001**. Helper T cell anergy: from biochemistry to cancer pathophysiology and therapeutics. *J Mol Med.* 78: 673-83
3. Arnt CR, Chiorean MV, Heldebrant MP, **2002**. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. *J Biol Chem.* 277: 44236-43
4. Ashkenazi A, Herbst RS, **2008a**. To kill a tumor cell: the potential of proapoptotic receptor agonists. *J Clin Invest.* 118: 1979-90
5. Ashkenazi A, Holland P, Eckhardt SG, **2008b**. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). *J Clin Oncol.* 26: 3621-30
6. Ashkenazi A, **2008c**. Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nat Rev Drug Discov.* (12): 1001-12
7. Ashkenazi A, Pai RC, Fong S, **1999**. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest.* 104: 155-62
8. Ashkenazi AJ, **2000**. Apo-2 ligand. US6030945
9. Ashkenazi AJ, Chuntharapai A, Kim KJ, **2001**. Method for making monoclonal antibodies and cross-reactive antibodies obtainable by the method. US6252050
10. Bastard JP, Jardel C, Bruckert E, **2000**. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab.* 85: 3338-42
11. Battaglia M, Stabilini A, Draghici E, **2006**. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes.* 55: 40-9
12. *Bernardi S, Norcio A, Toffoli B, 2012b. Potential role of TRAIL in the management of autoimmune diabetes mellitus. Curr Pharm Des.* 18: 5759-65
13. *Bernardi S, Secchiero P, Zauli G, 2012a. State of art and recent developments of anti-cancer strategies based on TRAIL. Rec Pat Anticancer Drug Discov.* 123: 29-37
14. *Bernardi S, Zauli G, Tikellis C, 2012c. TNF-related apoptosis-inducing ligand significantly attenuates metabolic abnormalities in high-fat-fed mice reducing adiposity and systemic inflammation. Clin Sci.* 123: 547-555
15. Bottazzo GF, Mann JI, Thorogood M, **1978**. Autoimmunity in juvenile diabetics and their families. *Br Med J.* 2: 165-168
16. Brimmell M, Mendiola R, Mangion J, **1998**. BAX frameshift mutations in cell lines derived from human haemopoietic malignancies are associated with resistance to apoptosis and microsatellite instability. *Oncogene.* 16: 1803-12
17. Brooks AD, Ramirez T, Toh U, **2005**. The proteasome inhibitor bortezomib (Velcade) sensitizes some human tumor cells to Apo2L/TRAIL-mediated apoptosis. *Ann N Y Acad Sci.* 1059: 160-7
18. Buschard K, Madsbad S, Rygaard J, **1980**. Depressed suppressor cell activity in patients with newly diagnosed insulin-dependent diabetes mellitus. *Clin Exp Immunol.* 41: 25-32
19. Calcutt NA, Cooper ME, Kern TS, **2009**. Therapies for hyperglycemia-induced diabetic complications: from animal models to clinical trials. *Nat Rev Drug Discov.* 8: 417-29
20. Camellia WA, **2006**. DR5 antibodies and uses thereof. US20060269554
21. Camidge DR, Herbst RS, Gordon MS, **2010**. A phase I safety and pharmacokinetic study of the death receptor 5 agonistic antibody PR095780 in patients with advanced malignancies. *Clin Cancer Res.* 16: 1256-63
22. Campioni D, Secchiero P, Corallini F, **2005**. Evidence for a role of TNF-related apoptosis-inducing ligand (TRAIL) in the anemia of myelodysplastic syndromes. *Am J Pathol.* 166: 557-563
23. Chai J, Du C, Wu JW, **2000**. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature.* 406: 855-62
24. Chatzigeorgiou A, Halapas A, Kalafatakis K, **2009**. The use of animal models in the study of diabetes mellitus. *In vivo.* 23:245-258
25. Choi JW, Song JS, Pai SH, **2004**. Associations of serum TRAIL concentrations, anthropometric variables, and serum lipid parameters in healthy adults. *Ann Clin Lab Sci.* 34: 400-404
26. Chuntharapai A, Dodge K, Grimmer K, **2001**. Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4. *J Immunol.* 166: 4891-8
27. Collins GS, Mallett S, Omar O, **2011**. Developing risk predictor models for type 2 diabetes: a systematic review of methodology and reporting. *BMC Med.* 8: 103-117
28. Cretney E, Takeda K, Yagita H, **2002**. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol.* 168: 1356-61
29. Croce CM, **2008**. Oncogenes and cancer. *N Engl J Med.* 358: 502-11
30. Cuello M, Etenberg SA, Nau MM, **2001**. Synergistic induction of apoptosis by the combination of trail and chemotherapy in chemoresistant ovarian cancer cells. *Gynecol Oncol.* 81: 380-90
31. Daniel D, Yang B, Lawrence DA, **2007**. Cooperation of the proapoptotic receptor agonist rhApo2L/TRAIL with the CD20 antibody rituximab against non-Hodgkin lymphoma xenografts. *Blood.* 110: 4037-46
32. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG, **1997b**. The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity.* 7: 813-20

33. Degli-Esposti MA, Smolak PJ, Walczak H, **1997a**. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med*. 186: 1165-70
34. Descamps FJ, Martens E, Ballaux F, **2004**. In vivo activation of gelatinase B/MMP-9 by trypsin in acute pancreatitis is a permissive factor in streptozotocin-induced diabetes. *J Pathol*. 204: 555-61
35. Di Bartolo BA, Chan J, Bennett MR, **2011**. TNF-related apoptosis inducing ligand (TRAIL) protects against diabetes and atherosclerosis in ApoE<sup>-/-</sup> mice. *Diabetologia*. 54: 3157-3167
36. Di Gregorio GB, Yao-Borengasser A, Rasouli N, **2005**. Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues. *Diabetes*. 54: 2305-2313
37. Di Lorenzo TP, Peakman M, Roep BO, **2007**. Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes. *Clin Exp Immunol*. 148:1-16
38. Di Pietro R, Zauli G, **2004**. Emerging non-apoptotic functions of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L. *J Cell Physiol*. 201: 331-40
39. Dirice E, Kahraman S, Elpek GO, **2011**. TRAIL and DcR1 expressions are differentially regulated in the pancreatic islets of STZ- versus CY-applied NOD mice. *Exp Diabetes Res*. 2011: 625813
40. Doi T, Murakami H, Ohtsu A, **2011**. Phase 1 study of conatumumab, a pro-apoptotic death receptor 5 agonist antibody, in Japanese patients with advanced solid tumors. *Cancer Chemother Pharmacol*. 68: 733-41
41. Donehower LA, Harvey M, Slagle BL, **1992**. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*. 356: 215-21
42. Dupre J for The Canadian-European Randomized Control Trial Group, **1988**. Cyclosporin-induced remission of IDDM after early intervention: association of 1 yr of cyclosporin treatment with enhanced insulin secretion. *Diabetes*. 37: 1574-1582
43. Ehrhardt H, Fulda S, Schmid I, **2003**: TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. *Oncogene*. 22: 3842-52
44. Eisenbarth GS, **1986**. Type 1 diabetes mellitus. *N Engl J Med*. 314: 1360-8
45. Esposito K, Pontillo A, Di Palo C, **2003**. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *JAMA*. 289: 1799-1804
46. Feutren G, Papoz L, Assan R, **1986**. Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset: results of a multicentre double-blind trial. *Lancet*. 2: 119-24
47. Forero-Torres A, Shah J, Wood T, **2010**. Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). *Cancer Biother Radiopharm*. 25: 13-9
48. Frese S, Brunner T, Gugger M, **2002**. Enhancement of Apo2L/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in non-small cell lung cancer cell lines by chemotherapeutic agents without correlation to the expression level of cellular protease caspase-8 inhibitory protein. *J Thorac Cardiovasc Surg*. 123: 168-74
49. Friesen C, Fulda S, Debatin KM, **1997**. Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. *Leukemia*. 11: 1833-41
50. Friesen C, Herr I, Krammer PH, **1996**. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med*. 2: 574-7
51. Fulda S, Wick W, Weller M, **2002**. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med*. 8: 808-15
52. Garofalo M, Di Leva G, Romano G, **2009**. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell*. 16: 498-509
53. Georgakis GV, Li Y, Humphreys R, Andreeff M, **2005**. Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. *Br J Haematol*. 130: 501-10
54. Gepts W, **1965**. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes*. 14: 619-33
55. Greco FA, Bonomi P, Crawford J, **2008**. Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer. *Lung Cancer*. 61: 82-90
56. Guo F, Sigua C, Tao J, **2004**. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death inducing signaling complex activity and apoptosis of human acute leukemia cells. *Cancer Res*. 64: 2580-9.
57. Hamilton-Williams EE, Palmer SE, Charlton B, **2003**. Beta cell MHC class I is a late requirement for diabetes. *Proc Natl Acad Sci USA*. 100: 6688-93
58. Han X, Sun Y, Scott S, Bleich D, **2001**. Tissue inhibitor of metalloproteinase-1 prevents cytokine-mediated dysfunction and cytotoxicity in pancreatic islets and b-cells. *Diabetes*. 50: 1047-55
59. Hanahan D, Weinberg RA, **2000**. The hallmarks of cancer. *Cell*. 100: 57-70
60. Harjutsalo V, Sjoberg L, Tuomilehto J, **2008**. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet*. 371: 1777-82
61. Harper N, Farrow SN, Kaptein A, **2001**. Modulation of tumor necrosis factor apoptosis-inducing ligand- induced NF-kappa B activation by inhibition of apical caspases. *J Biol Chem*. 276: 34743-52
62. Herbst RS, Eckhardt SG, Kurzrock R, **2010a**. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol*. 28: 2839-46
63. Herbst RS, Kurzrock R, Hong DS, **2010b**. A first-in-human study of conatumumab in adult patients with advanced solid tumors. *Clin Cancer Res*. 16: 5883-91
64. Herold KC, Gitelman SE, Masharani U, **2005**. A single course of anti-CD3 monoclonal antibody hOKT3gamma1 (Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes*. 54: 1763-69

65. Hotamisligil GS, Spiegelman BM, **1994**. Tumor necrosis factor  $\alpha$ : a key component of the obesity-diabetes link. *Diabetes*. 43: 1271-1278
66. Hotte SJ, Hirte HW, Chen EX, **2008**. A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin Cancer Res*. 14: 3450-55
67. Hu CY, Rodriguez-Pinto D, Du W, **2007**. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J Clin Invest*. 117: 3857-67
68. Hunter I, Nixon GF, **2006**. Spatial compartmentalization of tumor necrosis factor (TNF) receptor 1-dependent signaling pathways in human airway smooth muscle cells. Lipid rafts are essential for TNF-alpha-mediated activation of RhoA but dispensable for the activation of the NF-kappaB and MAPK pathways. *J Biol Chem*. 281: 34705-15
69. Hylander BL, Pitoniak R, Penetrante RB, **2005**. The anti-tumor effect of Apo2L/TRAIL on patient pancreatic adenocarcinomas grown as xenografts in SCID mice. *J Transl Med*. 3:22
70. Ichikawa K, Liu W, Zhao L, **2001**. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med*. 7: 954-60
71. Ikeda T, Hirata S, Fukushima S, **2010**. Dual effects of TRAIL in suppression of autoimmunity: the inhibition of Th1 cells and the promotion of regulatory T cells. *J Immunol*. 185: 5259-67
72. Janssen EM, Droin NM, Lemmens EE, **2005**. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*. 434: 88-93
73. Jin H, Yang R, Fong S, **2004**. Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand cooperates with chemotherapy to inhibit orthotopic lung tumor growth and improve survival. *Cancer Res*. 64: 4900-5
74. Jin X, Wu XX, Abdel-Muneem Nouh MA, **2007**. Enhancement of death receptor 4 mediated apoptosis and cytotoxicity in renal cell carcinoma cells by subtoxic concentrations of doxorubicin. *J Urol*. 177: 1894-9.
75. Johnson TR, Stone K, Nikrad M, **2003**. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. *Oncogene*. 22: 4953-63.
76. Kang S Park SY, Lee HJ, **2010a**. TRAIL upregulates decoy receptor 1 and mediates resistance to apoptosis in insulin-secreting INS-1 cells. *Biochem Biophys Res Comm*. 396: 731-5
77. Kang S, Park EJ, Joe Y, **2010b**. Systemic delivery of TNF-related apoptosis-inducing ligand (TRAIL) elevates levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and prevents type 1 diabetes in nonobese diabetic mice. *Endocrinology*. 151: 5638-46
78. Kaufmann SH, Vaux DL, **2003**. Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene*. 22: 7414-30.
79. Keuper M, Asterholm I, Scherer P, **2011**. TRAIL (TNF related apoptosis inducing ligand) affects adipogenic differentiation and adipocyte metabolism. *Proceedings of the 50th Annual Meeting of the European Society for Paediatric Endocrinology*, 25-28 September, Glasgow, UK
80. Kim K, Fisher MJ, Xu SQ, **2000**. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res*. 6: 335-46
81. Kim SH, Ricci MS, El-Deiry WS, **2008**. Mcl-1: a gateway to TRAIL sensitization. *Cancer Res*. 68: 2062-4
82. Koezwarra K, Bonifacio E, Ziegler AG, **2004**. Transmision of maternal islet antibodies and risk of autoimmune diabetes in offspring of mothers with type 1 diabetes. *Diabetes*. 53: 1-4
83. Lamhamed-Cherradi SE, Zheng S, Tisch RM, **2003**. Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes. *Diabetes*. 52: 2274-8
84. Lancaster JM, Sayer R, Blanchette C, **2003**. High expression of tumor necrosis factor-related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. *Clin Cancer Res*. 9: 762-6
85. Lane DP, **1992**. Cancer. p53, guardian of the genome. *Nature*. 358: 15-6
86. Lehrke M, Lazar RA, **2005**. The many faces of PPARgamma. *Cell*. 123: 993-999
87. Leong S, Cohen RB, Gustafson DL, **2009**. Mapatumumab, an antibody targeting TRAIL-R1, in combination with paclitaxel and carboplatin in patients with advanced solid malignancies: results of a phase I and pharmacokinetic study. *J Clin Oncol*. 27: 4413-21
88. Li B, Sidhu SS, **2008**. DR5 antibodies and uses thereof. US20080248037
89. Li R, Ruttinger D, Urba W, **2004a**. Targeting and amplification of immune killing of tumor cells by pro-Smac. *Int J Cancer*. 109: 85-94
90. Li L, Thomas RM, Suzuki H, **2004b**. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science*. 305: 1471-4
91. Lincz LF, Yeh TX, Spencer A, **2001**. TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrows is not related to TRAIL receptor expression or prior chemotherapy. *Leukemia*. 15: 1650-7
92. Lorz C, Benito-Martin A, Boucherot A, **2008**. The death ligand TRAIL in diabetic nephropathy. *JASN*. 19: 904-14
93. Ludvigsson J, Krisky D, Casas R, **2012**. GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus. *N Engl J Med*. 366: 433-42
94. Ludvigsson J, Faresjo M, Hjorth M, **2008**. GAD treatment and insulin secretion in recent-onset type 1 diabetes. *N Engl J Med*. 359: 1909-20
95. Mader I, Wabitsch M, Debatin KM, **2010**. Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1- independent sensitization to TRAIL-induced apoptosis. *FASEB J*. 24: 1997-2009
96. Maier T, Beckers T, Baer T, **2011**. Novel tetrahydro-fused pyridines as histone deacetylase inhibitors. US20110021494
97. Marsters SA, Sheridan JP, Pitti RM, **1997**. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol*. 7: 1003-6
98. Martin S, Wolf-Eichbaum D, Duinkerken G, **2001**. Development of type 1 diabetes despite severe hereditary B-cell deficiency. *N Engl J Med*. 345: 1036-9

99. Maxwell PR, Timms PM, Chandran S, **2001**. Peripheral blood level alterations of TIMP-1, MMP-2 and MMP-9 in patients with type 1 diabetes. *Diabet Med.* 18: 777-80
100. McGarry JD, **2002**. Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes.* 51: 7-18
101. Meng XW, Lee SH, Dai H, **2007**. Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *J Biol Chem.* 282: 29831-46
102. Mi QS, Ly D, Lamhamedi-Cherradi SE, **2003**. Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice. *Diabetes.* 52: 1967-75
103. Michowitz Y, Goldstein E, Roth A, **2005**. The involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in atherosclerosis. *J Am Coll Cardiol.* 45: 1018-24
104. Milani D, Zauli G, Rimondi E, **2003**. Tumour necrosis factor-related apoptosis-inducing ligand sequentially activates pro-survival and pro-apoptotic pathways in SK-N-MC neuronal cells. *J Neurochem.* 86: 126-35
105. Mirandola P, Ponti C, Gobbi G, **2004**. Activated human NK and CD8+T cells express both TNF-related apoptosis inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. *Blood.* 104: 2418-24
106. Mitsiades CS, Treon SP, Mitsiades N, **2001**. TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood.* 98: 795-804
107. Mitsiades N, Poulaki V, Tseleni-Balafouta S, **2000**. Thyroid carcinoma cells are resistant to FAS-mediated apoptosis but sensitive to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res.* 60: 4122-9
108. Mizutani Y, Yoshida O, Miki T, **1999**. Synergistic cytotoxicity and apoptosis by Apo-2 ligand and adriamycin against bladder cancer cells. *Clin Cancer Res.* 5: 2605-2612
109. Mom CH, Verweij J, Oldenhuis CN, **2009**. Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study. *Clin Cancer Res.* 15: 5584-90
110. Morel J, Audo R, Hahne M, **2005**. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces rheumatoid arthritis synovial fibroblast proliferation through mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt. *J Biol Chem.* 280: 15709-18
111. Nagata S, **1997**. Apoptosis by death factor. *Cell.* 88: 355-65
112. Nerup J, Andersen OO, Bendixen G, **1974**. Cell-mediated autoimmunity in diabetes mellitus. *Proc R Soc Med.* 67: 506-10
113. Ozoren N, El-Deiry WS, **2002**. Defining characteristics of Types I and II apoptotic cells in response to TRAIL. *Neoplasia.* 4: 551-7
114. Pan G, Ni J, Wei YF, **1997b**. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science.* 277: 815-8
115. Pan G, O'Rourke K, Chinnaiyan AM, **1997a**. The receptor for the cytotoxic ligand TRAIL. *Science.* 276: 111-3
116. Pescovitz MD for Type 1 Diabetes TrialNet Anti-CD20 Study Group, **2009**. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med.* 361: 2143-52
117. Plummer R, Attard G, Pacey S, **2007**. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res.* 13: 6187-94
118. Pommier Y, Sordet O, Antony S, **2004**. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene.* 23: 2934-49
119. Rampino N, Yamamoto H, Ionov Y, **1997**. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science.* 275: 967-9
120. Ravi R, Jain AJ, Schulick RD, **2004**. Elimination of hepatic metastases of colon cancer cells via p53-independent cross-talk between irinotecan and Apo2 ligand/TRAIL. *Cancer Res.* 64: 9105-14
121. Ray S, Bucur O, Almasan A, **2005**. Sensitization of prostate carcinoma cells to Apo2L/TRAIL by a Bcl-2 family protein inhibitor. *Apoptosis.* 10: 1411-8
122. Raz I, Elias D, Avron A, **2001**. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomized, double-blind, phase II trial. *Lancet.* 358: 1749-53
123. Ricci MS, Kim SH, Ogi K, **2007**. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell.* 12: 66-80
124. Ricordi C, **2003**. Islet transplantation: a brave new world. *Diabetes.* 52: 1595-1603
125. Roep BO, **2003**. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. *Diabetologia.* 46: 305-321
126. Roncarolo MG, Battaglia M, **2007**. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol.* 7: 585-98
127. Rosato RR, Almenara JA, Coe S, **2007**. The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res.* 67: 9490-500.
128. Ryan AS, Nicklas BJ, **1994**. Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women. *Diabetes Care.* 27: 1699-1705
129. Sakaguchi S, Powrie F, Ransohoff RM, **2012**. Re-establishing immunological self-tolerance in autoimmune disease. *Nat Med.* 18: 54-8
130. Salcedo T, Roschke V, Rosen CA, **2003**. Antibodies that immunospecifically bind to TRAIL. US20030228309
131. Schoppet M, Sattler AM, Schaefer JR, **2006**. Osteoprotegerin (OPG) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) levels in atherosclerosis. *Atherosclerosis.* 184: 446-7
132. Secchiero P, Candido R, Corallini F, **2006**. Systemic tumor necrosis factor-related apoptosis-inducing ligand delivery shows antiatherosclerotic activity in apolipoprotein E-null diabetic mice. *Circulation.* 114: 1522-30
133. Secchiero P, Corallini F, Ceconi C, **2009**. Potential prognostic significance of decreased serum levels of TRAIL after acute myocardial infarction. *PLoS One.* 4: e4442

134. Secchiero P, Gonelli A, Carnevale E, **2003**. TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. *Circulation*. 107: 2250-6.
135. Secchiero P, Perri P, Melloni E, **2011**. Decreased levels of soluble TNF-related apoptosis-inducing ligand (TRAIL) in the conjunctival sac fluid of patients with diabetes affected by proliferative retinopathy. *Diabet Med*. 28: 1277-8
136. Secchiero P, Zauli G, **2008**. TNF-related apoptosis-inducing ligand and the regulation of hematopoiesis. *Curr Op Hematol*. 15: 42-8
137. Secchiero P, Zerbinati C, Rimondi E, **2004**. TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells. *Cell Mol Life Sci*. 61: 1965-74
138. Sedger LM, Glaccum MB, Schuh JC, **2002**. Characterization of the *in vivo* function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. *Eur J Immunol*. 32: 2246-54
139. Shapiro AM, Ricordi C, Hering BJ, **2006**. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. 355: 1318-30
140. Sheridan JP, Marsters SA, Pitti RM, **1997**. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*. 277: 818-21.
141. Shiraishi T, Suzuyama K, Okamoto H, **2004**. Increased cytotoxicity of soluble Fas ligand by fusing isoleucine zipper motif. *Biochem Biophys Res Commun*. 322: 197-202.
142. Song JH, Tse MC, Bellail A, **2007**. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. *Cancer Res*. 67: 6946-55
143. Soria JC, Smit E, Khayat D, **2010**. Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer. *J Clin Oncol*. 28: 1527-33
144. Tamm I, Wang Y, Sausville E, **1998**. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res*. 58: 5315-20
145. Tartaglia LA, Goeddel DV, **1992**. Two TNF receptors. *Immunol Today*. 13: 151-3
146. [Thomas M, Zauli G, Secchiero P, Fabris B, Bernardi S, 2012. Apoptosis-inducing molecules and uses therefor. \*WO 2012117336\*](#)
147. [Toffoli B, Bernardi S, Candido R, 2012. TRAIL shows potential cardioprotective activity. \*Invest New Drugs\*. 30: 1257-60](#)
148. Tolcher AW, Mita M, Meropol NJ, **2007**. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol*. 25: 1390-5.
149. Trarbach T, Moehler M, Heinemann V, **2010**. Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. *Br J Cancer*. 102: 506-12.
150. Tuomilhto for Diabetes Prevention Study Group, **2001**. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med*. 344: 1343-1350
151. Turner N, Bruce CR, Beale SM, **2007**. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes*. 56: 2085-2892
152. Unger RH, Orci L, **2001**. Disorders of liporegulation: new perspective on obesity and related disorders. *FASEB J*. 15: 312-321
153. Van Belle TL, Coppieters KT, Von Herrath MG, **2011**. Type 1 diabetes: etiology, immunology and therapeutic strategies. *Physiol Rev*. 91: 79-118
154. Verbrugge I, Wissink EH, Rooswinkel RW, **2009**. Combining radiotherapy with APO010 in cancer treatment. *Clin Cancer Res*. 15: 2031-8
155. Vignati S, Codegioni A, Polato F, **2002**. Trail activity in human ovarian cancer cells: potentiation of the action of cytotoxic drugs. *Eur J Cancer*. 38: 177-83
156. Wakelee HA, Patnaik A, Sikic BI, **2010**. Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors. *Ann Oncol*. 21: 376-81
157. Wiezorek J, Holland P, Graves J, **2010**. Death receptor agonists as a targeted therapy for cancer *Clin Cancer Res*. 16: 1701-8
158. Wiley SR, Goodwin RG, **2001**. Cytokine that induce apoptosis. *US6284236*
159. Wiley SR, Schooley K, Smolak PJ, **1995**. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 3: 673-82
160. Wu G, Chai J, Suber TL, **2000**. Structural basis of IAP recognition by Smac/DIABLO. *Nature*. 408: 1008-12.
161. Wu GS, Burns TF, McDonald ER, 3rd, **1997**. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet*. 17: 141-3
162. Wuchter C, Krappmann D, Cai Z, **2001**. In vitro susceptibility to TRAIL-induced apoptosis of acute leukemia cells in the context of TRAIL receptor gene expression and constitutive NF-kappa B activity. *Leukemia*. 15: 921-8
163. Xu H, Barnes GT, Yang Q, **2003**. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 112: 1821-1830
164. Xue M, Thompson PJ, Clifton-Bligh R, **2005**. Leukocyte matrix metalloproteinase-9 is elevated and contributes to lymphocyte activation in type 1 diabetes. *Int J Biochem Cell Biol*. 37: 2406-16
165. Yao GH, Ling LJ, Luan JF, **2007**. Induction of apoptosis by recombinant soluble human TRAIL in Jurkat cells. *Biomed Environ Sci*. 20: 470-7
166. Younes A, Vose JM, Zelenetz AD, **2010**. A Phase 1b/2 trial of mapatumumab in patients with relapsed/refractory non-Hodgkin's lymphoma. *Br J Cancer*. 103: 1783-87

167. Yu R, Mandlekar S, Ruben S, **2000**. Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in androgen-independent prostate cancer cells. *Cancer Res.* 60: 2384-9
168. Zangemeister-Wittke U, **2003**. Antisense to apoptosis inhibitors facilitates chemotherapy and TRAIL-induced death signaling. *Ann N Y Acad Sci.* 1002: 90-4
169. Zauli G, Melloni E, Capitani S, **2009**. Role of full-length osteoprotegerin in tumor cell biology. *Cell Mol Life Sci.* 66: 841-51.
170. Zauli G, Sancilio S, Cataldi A, **2005**. PI-3K/Akt and NF- $\kappa$ B/I $\kappa$ B $\alpha$  pathways are activated in Jurkat T cells in response to TRAIL treatment. *J Cell Physiol.* 202: 900-11.
171. Zauli G, Toffoli B, di Iasio MG, **2010**. Treatment with recombinant tumor necrosis factor-related apoptosis-inducing ligand alleviates the severity of streptozotocin-induced diabetes. *Diabetes.* 59: 1261-5
172. Zhu H, Guo W, Zhang L, **2005**. Proteasome inhibitors-mediated TRAIL resensitization and Bik accumulation. *Cancer Biol Ther.* 4: 781-6.

