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Angiopoietin-like proteins as therapeutic targets for cardiovascular disease: focus on lipid disorders

Marco Bruno Morelli[1,2], **Christopher Chavez**[1], **Gaetano Santulli**[1,2,3]

[1]Department of Medicine; Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, New York, NY, USA

[2]Department of Molecular Pharmacology, Einstein-Mount Sinai Diabetes Research Center (ES-DRC), The “Norman Fleischer” Institute for Diabetes and Metabolism, Albert Einstein College of Medicine, New York, NY, USA

[3]Department of Advanced Biomedical Sciences, “Federico II” University, Naples, Italy.

Abstract

Introduction: Angiopoietin-like (ANGPTL) proteins belong to a family of eight secreted factors that are structurally related to proteins that modulate angiogenesis; these are commonly known as angiopoietins. Angiopoietin-like proteins, ANGPTL3, ANGPTL4, and ANGPTL8 (the “ANGPT L3–4-8 triad”), have surfaced as principal regulators of plasma lipid metabolism by functioning as potent inhibitors of lipoprotein lipase. The targeting of these proteins may open up future therapeutic avenues for metabolic and cardiovascular disease.

Areas covered: This article systematically summarizes the compelling literature that describes the mechanistic roles of ANGPTL3, 4, and 8 in lipid metabolism; this emphasizes their importance in determining the risk of cardiovascular disease. We shed light on population-based studies linking loss-of-function variations in ANGPTL3, 4, and 8 with decreased risk of metabolic conditions and cardiovascular disorders. We also discuss how the targeting of the ANGPT L3–4-8 triad could one day offer therapeutic benefit.

Expert opinion: Monoclonal antibodies and antisense oligonucleotides that target ANGPTL3, 4, and 8 are potentially an efficient therapeutic strategy for hypertriglyceridemia and cardiovascular risk reduction, especially in patients with limited treatment options. These innovative therapeutical approaches are at an embryonic stage in development and hence further investigations are necessary for eventual use in humans.

Corresponding author: Gaetano Santulli, gaetano.santulli@einstein.yu.edu.

Declaration of interest

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angiopoietin; ANGPTL; ANGPTL3-4-8 triad; cardiovascular disease; cholesterol; diabetes mellitus; evinacumab; LPL; metabolic syndrome obesity; PCSK9; REGN3776; triglycerides

1. Introduction

Angiopoietin-like (ANGPTL) proteins, a superfamily of secreted proteins structurally related to factors modulating angiogenesis, known as angiopoietins. This family includes eight proteins members (Table 1) encoded by eight genes (ANGPTL1–8). Three members of this family – namely ANGPTL3, ANGPTL4, and ANGPTL8 – have been largely studied for their role as crucial regulators of lipid metabolism in heart, skeletal muscles, white adipose tissue (WAT), and brown adipose tissue (BAT) [1–4]. Specifically, these ANGPTL proteins have been shown to antagonize the hydrolysis of triglycerides (TGs) mediated by lipoprotein lipase (LPL). Mounting evidence suggests that the modulation of these three ANGPTL proteins is promising in decreasing the levels of circulating lipoproteins, as well as reducing the risk of cardiovascular disease.

2. LPL, TG metabolism, and cardiovascular disease

TGs represent one of the main forms of lipids to store and provide energy. TGs are emulsified by proteins to form lipoproteins, carriers for hydrophobic lipid molecules in the bloodstream; chylomicrons and Very-Low-Density Lipoprotein (VLDL) are the two major TG-rich classes of lipoproteins. These lipoproteins transport and distribute TGs to various tissues, including liver, adipose tissue, and muscles; in the capillaries of these tissues, the partial hydrolysis of the core TGs of chylomicrons and VLDL to monoglycerides and fatty acids is catalyzed by a single type of enzyme, the lipase [5].

LPL belongs to the TGs lipase gene family and is the major lipolytic enzyme involved in the intravascular metabolism of TGs-rich lipoproteins [6]. LPL is expressed in the heart, skeletal muscles, WAT and BAT, but not in capillary endothelial cells [7]. Adipocytes and (cardio)myocytes express and secrete LPL, which is transported as dimer across the endothelial cells of capillaries, where the active LPL is exposed and anchored to the luminal surface [7].

The trans-endothelial transport of LPL towards the capillary lumen is mediated by Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1), which has a high binding affinity for LPL [7–9]. The Heparan Sulfated Peptidoglycan (HSPG) binds LPL in the interstitial space, allowing the interaction with GPIHBP1 [10]. Numerous studies based on protein lipase X-ray crystallography and molecular modeling were conducted from the middle of the ‘90s until now: Van Tilbeurgh et al. in 1994 published the first LPL structural model obtained from pancreatic lipase crystals [11]; LPL in its activated state is a 55-kDa noncovalently-bound homodimer with a head-to-tail orientation [8, 12]. The hydrolysis of TGs present in TG-rich lipoproteins occurs through the interaction with Apolipoprotein C2, a key component of both VLDL and chylomicrons [13]. Specifically, modeling studies indicate that the catalytic site of LPL – a conserved

Ser^{132}, Asp^{156}, and His^{241} sequence in the N-terminal domain – is covered by a mobile subdomain lid (or flap) [11, 14] that controls catalytic activity and can be opened by the binding of chylomicrons and VLDL to the C-terminus of LPL [15]. Recently, Birrane et al. have shown the structure of LPL complexed with GPIHBP1 using X-ray crystallography [12]. Such analysis has permitted to define how GPIHBP1 interacts, anchors, and stabilizes LPL [12]. This crystallography study confirmed the observations of Reimund and collaborators about two distinct binding sites for LPL on GPIHBP1 with conserved LPL-lipoproteins interaction in presence of the LPL binding to the GPIHBP1's acidic peptides corresponding to the N-terminal domain [16]. Successively, experiments performed in Michael Ploug's laboratory demonstrated that LPL binding to the GPIHBP1's N-terminal domain prevents the LPL unfolding [17, 18]. More recently, the properties of GPIHBP1's Intrinsically Disordered Region (IDR) within the N-terminal domain were defined by the same group of investigators: the tyrosine O-sulfation in the IDR increases both the affinity of GPIHBP1-LPL interactions and the ability of GPIHBP1 to protect LPL against the ANGPTL4-induced unfolding [19]; moreover, the negative charges of the acidic residues in GPIHBP1's IDR facilitate the capture of LPL by capillary endothelial cells, thereby contributing to GPIHBP1's ability to preserve LPL structure and activity [19]. For decades, the homodimeric conformation of LPL was assumed to be the only one capable of hydrolyzing TGs, with a loss of enzymatic activity when the LPL dimer is converted into inactive folded monomers [20]. Lookene and colleagues observed that LPL dimeric form can rapidly exchange the monomeric subunits, indicating a spontaneous dynamic equilibrium between active LPL dimers and dimerization-competent monomers [21]. Whether the monomeric conformation can be catalytically active remained unclear until a recent study revisited the consolidated notion that LPL is active only as a homodimer, showing that a catalytically active form of LPL can also exist in a monomeric state [22].

Substantial evidence has shown that hypertriglyceridemia and loss-of-function (LoF) LPL variants act as independent risk factors for coronary artery disease (CAD), although a reduced LPL activity entails per se the elevation of circulating TGs levels [23–25]. A strong correlation between TGs levels and CAD was provided by a study involving 13953 subjects (26–45 years old) followed up for ~10.5 years: TGs levels in the top quintile at baseline were associated with a 4-fold increased risk of CAD compared with the lowest TGs quintile, confirmed after adjustment for other risk factors, including HDL-C [24]; case-control and angiographic studies have confirmed that elevated TGs levels represent a risk factor for CAD [26].

Genome-wide association studies (GWAS) have revealed that common noncoding variants within the LPL gene locus associated with both elevated TGs levels and CAD risk [27, 28]. In a study conducted on 773 men recruited in the United Kingdom, Mailly et al. showed a significant correlation between LPL LoF polymorphisms and combined hyperlipidemia; the most common LPL variant was the substitution of Asp to Asn in position 9 (D9N) [29]. More recently, Stitziel et al. identified a low-frequency missense variant (D36N) in LPL that increases the risk of CAD [30]. On the other hand, various studies identified a gain-of-function LPL (truncated variant S447^{x}), associated with lower levels of circulating TGs and lower incidence of vascular disease or myocardial infarction compared to non-carriers [25, 31–35].

3. ANGPTL3, 4, and 8 as circulating TG regulators

3.1. Overview of the ANGPTL3–4-8 triad

The ANGPTL3–4-8 triad balances the TG uptake in WAT and oxidative tissues (muscle) by a finely tuned regulation of LPL activity in different nutritional states [1, 36, 37].

ANGPTL3 was discovered in 1999 by Darrell Conklin and collaborators [38] and is considered a potent modulator of TGs, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). In humans, ANGPTL3 is a 70 kDa protein (54 KDa before glycosylation) predominantly produced in the liver [23, 38–40]. ANGPTL3 has been shown to promote the flow of lipoprotein-derived fatty acids to WAT for storage during the fed state by reducing the activity of LPL in muscle and heart [41]. ANGPTL3 inhibits endothelial lipase [42–44], a member of the TG lipase gene family with a considerable molecular homology with LPL (44%) and hepatic lipase (HL) (41%), but unlike LPL and HL, it is synthesized by endothelial cells [45, 46].

ANGPTL4 protein was described a year later, in 2000, by three independent research groups that simultaneously identified this molecule as a fasting-induced adipose factor and potent LPL inhibitor [47–49]. Mainly expressed in liver and adipose tissue, ANGPTL acts as a potent switch of TGs homeostasis, causing reduced uptake of fatty acids from TG-rich lipoproteins in WAT during fasting and physical activity [50]. ANGPTL4 expression increases in muscle during fasting and is involved in a fine modulation of energy expenditure by selective inhibition of LPL in resting muscles [51, 52]. Catoire and collaborators observed that during endurance exercise the selective induction of ANGPTL4 in the non-exercising muscle reduces local fatty acid uptake presumably to prevent TGs overload, thereby directing fatty acids towards the actively contracting muscles for energy production [52]. Protein sequence analyses revealed that, among the members of ANGPTL family, ANGPTL3 is the only one that shares a high percentage of similarity with the LPL binding core of ANGPTL4 [53]; mapping ANGPTL3 and −4 protein sequences confirmed the presence of an analogous domain essential for the direct binding of LPL dimer [53].

Only in 2012, a new feeding-induced hepatokine has been identified: originally called lipasin, RIFL, and betatrophin [1, 54, 55], the official name of the protein as ANGPTL8 was established in 2015 by the Human Genome Organisation (HUGO) Gene Nomenclature Committee. ANGPTL8 is mostly produced in liver and WAT, its protein structure lacks the fibrinogen-like domain, and by itself has no detectable effect on LPL; the ANGPTL8 inhibition of LPL activity occurs only in presence of ANGPTL3, which exhibits an augmented inhibitory effect on LPL in muscle and heart following its direct interaction with ANGPTL8 [56–59]. Furthermore, ANGPTL8 inhibition via a human monoclonal antibody (REGN3776) decreased plasma TGs levels and increases LPL activity in humanized-ANGPTL8 mice, proving that despite not being able to directly bind LPL, ANGPTL8 induces a strong indirect inactivation on LPL activity [60]. Interestingly, in a recent work, Kovrov et al. reported that ANGPTL8 can form complexes with ANGPTL3 and with ANGPTL4, thereby acting as a critical metabolic switch [56].

3.2. ANGPTL3 and 4: structural organization and mechanisms of action

The first indication of the involvement of ANGPTL in lipid metabolism originated from a subgroup of KK/San mice, displaying extremely low TG levels; a LoF mutation in ANGPTL3 was identified in these mice, suggesting that the low TG level could be somehow attributable to ANGPTL3 deficiency [42]. Upon secretion in the bloodstream, ANGPTL3 activates lipolysis in the adipose tissue, increasing the release of fatty free acids and glycerol from adipocytes, and inhibits LPL activity in muscles and heart, increasing circulating TG-rich lipoproteins [43]. ANGPTL3 shares with the other ANGPTL members an N-terminal α-helix region predicted to fold into a coiled-coil structure and a C-terminal fibrinogen-like domain, as well as a 16-aa signal peptide required for secretion [61].

As mentioned above, ANGPTL4 was initially identified as an adipokine exclusively involved in lipid metabolism, given its considerable expression in adipose tissue [47, 48]; however, ANGTL4 is also expressed in the liver and secreted in circulation [56]. This protein regulates lipid metabolism by inhibiting LPL activity and stimulating lipolysis of WAT, resulting in increased levels of plasma TGs and fatty acids [56, 62, 63]. Angptl4 gene encodes for a native full-length glycoprotein (45–65 kDa) that, similarly to ANGPTL3, consists of an N-terminal coiled-coil domain involved in LPL inhibition and a C-terminal fibrinogen-like domain, as well as a signal peptide required for secretion; a linker region between N-terminal and C-terminal domains is required for the activation of ANGPTL3 and ANGPTL4 [62, 64].

In the liver, the activation of ANGPTL3 and −4 is triggered by proprotein convertases subtilisin/kexins (PCSK3, PCSK4, PCSK5, PACE4, and PCSK7) that cleave the recognition sequence in the linker region, releasing the N-terminal domain, which has been shown to have a markedly higher LPL inhibition efficiency than the entire ANGPTL3 and −4 protein sequences [65].

The molecular mechanisms underlying ANGPTL3 and ANGPTL4 mediated inhibition of LPL are still debated. Different authors have demonstrated that the N-terminal coiled-coil region of ANGPTL3 and −4 binds and irreversibly inactivates LPL, promoting the dissociation of lipase dimers to monomers [53, 56]. Specifically, in a recent work based on the computational model of dimeric LPL, the key binding sites for ANGPTL4 were identified in the LPL lid and lid-proximal helix [66]. Mapping the ANGPTL3 and ANGPTL4 protein sequences, Lee and collaborators identified in their N-terminal domains the epitope, designated as specific epitope 1, necessary to directly bind lipases and inactivate their enzymatic activity [53]. Nevertheless, liver-derived ANGPTL3 has been shown to enhance the cleavage of LPL by acting as PCSKs cofactor: such effect is specific for LPL and does not involve endothelial lipases [67]; both N- and C-terminal domains of LPL are required for ANGPTL3-enhanced cleavage, and the N-terminal domain of ANGPTL3 is sufficient to exert its effect on LPL cleavage [67]. Moreover, ANGPTL3 enhances LPL cleavage in the presence of either HSPG or GPIHBP1 [67]. Since ANGPTL3 and LPL are not produced in the same tissues, the effect of ANGPTL3 on PCSK-mediated LPL cleavage is likely extracellular, in a system in which LPL is released from the endothelial cell surface.

While ANGPTL3 is secreted in the bloodstream and inhibits the activity of LPL in heart and skeletal muscle, ANGPTL4 is a circulating protein released from the liver, but it has been also proposed to be a local regulator of LPL activity via an autocrine and/or paracrine action, in particular in the adipose tissue [68]. Dijk and colleagues demonstrated that ANGPTL4 interacts with LPL even before its secretion, at least in adipocytes, leading to LPL intracellular inactivation and degradation through PCSKs-mediated cleavage [65, 69]. Therefore, lipase inactivation might occur through a direct binding of the LPL dimer to the entire un-cleaved ANGPTL3 and 4 or relative cleaved N-terminal domain, with consequent LPL inactivation by enzymatic dimer dissociation [53]. Nevertheless, some studies suggest that ANGPTL3 on the endothelial cell surface, as well as ANGPTL4 both in the intracellular and extracellular side, may unfold and dissociate the lipase dimer, unmasking in LPL the PCSKs cleavage site (at residues 321–324), thereby rendering LPL more susceptible to cleavage and subsequent degradation [18, 20, 65].

3.3. ANGPTL3 and 4: transcriptional regulation

At the transcriptional level, ANGPTL3 is mainly regulated by the Liver X receptor (LXR) and Hepatocyte Nuclear Factor 1α (HNF-1α) pathways [40, 67, 70]. LXRs play a pivotal role in cholesterol homeostasis and fatty acid metabolism by inducing the transcription of sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase, and LPL. HNF-1α is known to be a key transcriptional factor of PCSK9, and to promote transcription in a coordinated manner with SREBP-1c [71, 72]. Moreover, insulin decreases the expression of HNF-1α by activation of the PI3K/PKB/Akt signaling pathway through the mammalian target of rapamycin complex 1 (mTORC-1) pathway [73]. Yki-Järvinen's group reported that insulin decreases ANGPTL3 plasma levels in humans, whereas in immortalized human hepatocyte cells insulin decreases ANGPTL3 gene and protein expression [40]. These data are consistent with the assays performed by Shimamura and collaborators, who observed a downregulation of ANGPTL3 mRNA and relative protein expression in human hepatoma HepG2 cells induced by insulin treatment [43]. In previous experiments, Inukai and colleagues had observed that ANGPTL3 mRNA was increased approximately 2.2-fold in the livers of streptozotocin-treated mice, and this effect was dramatically reversed by the administration of insulin [74]; in the same study, ANGPTL3 mRNA was found to be increased more than 3-fold in type 2 diabetes mellitus (T2DM) obese mice compared with age-matched lean littermates; the hepatic level of ANGPTL3 protein was also augmented in these diabetic mice to an extent similar to that of ANGPTL3 mRNA [74]. Another factor that negatively regulates ANGPTL3 expression is leptin: indeed, plasma ANGPTL3 levels are increased both in leptin-resistant (db/db) and leptin-deficient (ob/ob) mice, relative to wild-type mice; treatment with leptin directly suppresses ANGPTL3 expression in hepatocytes and plasma ANGPTL3 in ob/ob mice [43].

ANGPTL4 expression is regulated by several transcriptions factors, including PPARs nuclear receptors (PPARα and PPARγ) and HIF-1α; additionally, upregulation of ANGPTL4 is strongly linked to fasting state [75]. Several molecular actors that mediate the fasting effects seem to increase ANGPTL4. Some observations indicates that glucocorticoids, whose circulating levels are high during fasting, might increase ANGPTL4 expression [76, 77]. Koliwad and collaborators showed that the synthetic glucocorticoid

dexamethasone increases ANGPTL4 mRNA levels in primary hepatocytes and adipocytes (2–3-fold), but also in the liver and WAT of treated mice (approximately 4-fold) [77]; they also identified a glucocorticoid receptor (GR) binding site within the rat Angptl4 gene sequence, and these GR binding site sequences are conserved in the human and mouse genome [77]. Furthermore, a study conducted on human myofibroblasts using genome-wide transcriptional profiling technology revealed that human ANGPTL4 expression might be synergistically induced by the functional interactions of TGF-β and PPARβ/δ signaling [78]. Most recently, a regulation of ANGPTL transcription by microRNAs, has also been reported [79].

3.4. ANGPTL8: structural organization and mechanisms of action

ANGPTL8 was included in the family of ANGPTL proteins in 2012, and its crucial function in the control of lipid metabolism was rapidly recognized [54, 55]. Structurally, ANGPTL8 is considered an atypical member since its protein sequence presents only the N-terminal region (paralog of the N-terminal domain) but lacks the main structural features present in all other proteins of the group, such as the C-terminal fibrinogen-like domain, glycosylation sites, and amino acids requested for disulfide bonds formation [1, 62, 80].

ANGPTL8 is mainly produced in liver and WAT and is released into the bloodstream; by itself, it has no detectable effect on LPL activity inasmuch as it is not able to bind directly LPL nor EL [56]. However, several in vivo studies in murine models revealed a clear association between ANGPTL8 levels and modifications of the lipid profile. Overexpressing ANGPTL8 in the mouse liver using recombinant adenovirus injection led to dramatically increased serum TGs levels [55]. On the other hand, in mice lacking ANGPTL8, the postprandial increase in VLDL-TG uptake by WAT was abolished and the suppression of lipolysis that occurs in WAT with refeeding was markedly impaired; despite major alterations in TG metabolism, no defects in glucose metabolism were detected in ANGPTL8$^{-/-}$ mice [81]. Overexpressing ANGPTL8 was shown to increase serum TGs levels in an ANGPTLl3-dependent manner [1]; similarly, ANGPTL8 KO mice exhibit higher LPL activity specifically in cardiac and skeletal muscles in the fed state, suggesting that ANGPTL8 is required for LPL inhibition in these tissues [82]. Altogether these results indicate that ANGPTL8 negatively regulates LPL activity in the heart and in the skeletal muscle, and reduction of LPL activity by ANGPTL8 occurs only in the presence of ANGPTL3 [57, 59]. Intriguingly, ANGPTL8 has been also shown to act as a key regulator of the liver clock in response to food [83].

Two major mechanisms have been proposed to explain the ANGPTL8-mediated enhancement of lipase-inhibition by ANGPTL3: a cleavage at the linker region of ANGPTL that releases the relative N-terminal domain, which in turn targets LPL in the striated muscle (in this scenario, ANGPTL8 itself remains in the circulation); the formation of a complex between ANGPTL3 and ANGPTL8, that translocates to muscle capillaries where it inhibits LPL [56]. The latter scenario seems more probable, since ANGPTL8 KO mice do not exhibit reduced circulating levels of ANGPTL3 N-terminal domain [81], and thus ANGPTL8 does not appear to be required for ANGPTL3 cleavage. Moreover, in mice with ANGPTL8 overexpression, levels of circulating ANGPTL3 are reduced [1], supporting the

notion that exogenous ANGPTL8 is able to form complexes with ANGPTL3 [1, 84]. This mechanism explains both the absent reduction of ANGPTL3 N-terminal domain in ANGPTL8$^{-/-}$ mice and the reduction of circulating ANGPTL3 when ANGPTL8 is overexpressed.

3.5. ANGPTL8: transcriptional regulation

Fu et al. identified within the Angptl8 gene the presence of carbohydrate response elements (ChRE) [85]; the ChRE binding protein (ChREBP), a glucose-induced transcription factor involved in energy homeostasis, recognizes ChRE element and plays a significant role in both glucose and lipid metabolism [85]. Rong Guo and colleagues using transgenic human liver hepatocellular lines (HepG2) overexpressing or ablating ANGPTL8, proposed a model of ANGPTL8 activation via insulin signaling pathway, in which ANGPTL8 helps to ameliorate insulin resistance by enhancing the glucose-lowering effect through insulin-stimulated activation of the Akt-GSK3β or Akt-FoxO1 pathway, in a manner independent from the presence of a condition of insulin resistance [86].

The expression of ANGPTL8 is also promoted in the liver by LXRα; indeed, ANGPTL8 expression is significantly increased in HepG2 cells exposed to T0901317 (an established LXR agonist); such effect is reversed when cells are treated with AICAR, a permeable activator of 5’ adenosine monophosphate (AMP)-activated protein kinase (AMPK) [87]. The inhibitory effects of AICAR on the T0901317-induced ANGPTL8 expression are especially evident after SREBP silencing in cells that have been transfected with SREBP-1c siRNA [87]; this finding strongly suggests that SREBP-1c transcription factor promotes ANGPTL8 expression. Additionally, AICAR increases PPARα phosphorylation likely through AMPK, and the AICAR effect is not observed in HepG2 cells treated with PPARα inhibitor. Metformin, a drug known to activate AMPK, shows effects on ANGPTL8 similar to those of AICAR [87]. These data suggest that AMPK, via PPARα phosphorylation, can inhibit the expression of LXR/SREBP-1 transcription factors, also during T0901317 induction, and such inhibition suppresses ANGPTL8 expression.

LXR is also involved in the crosstalk with other nuclear receptors, including thyroid hormone receptor; the induction of thyroid hormone receptors by thyroid hormone was reported to up-regulate the expression of ANGPTL8 in human liver cell lines [88], interestingly, intracellular thyroid hormone activation is itself under the control of nutritional stimulus [89]. ANGPTL8 has been recently identified as a target of specific miRNAs, including 143–3p and 221–3p [90, 91], in diverse cell types.

4. ANGPTL3–4-8: orchestrators of LPL-mediated TG uptake

After a meal, TGs are directed to WAT for storage and to the heart and skeletal muscle for oxidation, in order to generate energy during fasting or prolonged physical exercise. The processes of TGs trafficking are critically coordinated by LPL; in fact, in the fed state, LPL activity rises in WAT but declines in muscles; conversely, during the fasting state or physical exercise, LPL activity declines in WAT but rises in muscles [92].

Initially, based only on ANGPTL3 and ANGPTL4, the LPL regulation among WAT, heart, and skeletal muscle could not be completely explained. When the third member in the TG regulation, now known as ANGPTL8, was identified, the TGs regulation model became more clear [58]. TG trafficking is mainly directed towards two different kinds of tissues, adipose tissue for TG storage after a meal, or skeletal and cardiac muscle for oxidative metabolism during the fasting state or physical exercise. To switch the energy homeostasis toward one tissue or the other, ANGPTL proteins finely orchestrate the regulation of LPL in adipose tissue or muscle. Furthermore, levels of ANGPTL4 and ANGPTL8 are strictly dependent on the nutritional state; instead, ANGPTL3 seems to remain fairly stable in the bloodstream, since its level does not depend on the nutritional state [1, 93]. However, by itself, ANGPTL3 shows a relatively weak inhibition of LPL, therefore it needs to be associated with ANGPTL8 to increase the inhibitory effect on LPL [56]. During fasting conditions or physical exercise, ANGPTL4 reaches its maximal plasma level, while ANGPTL8 is not synthesized. Under these conditions, ANGPTL4 binds to LPL in WAT, inhibiting the TGs hydrolyzing activity [94]. Furthermore, during fasting or physical exercise, the LPL in capillaries of cardiac and skeletal muscles are not inhibited by ANGPTL3–8, since ANGPTL8 is not expressed in this nutritional state; therefore muscles and heart can uptake TGs mobilized from WAT to satisfy the oxidative metabolism necessities; indeed, after a meal ANGPTL8 plasma levels increase and ANGPTL4 levels dramatically decrease [94]. ANGPTL8 binds ANGPTL3 to form a heterodimer which then binds and inhibits LPL in both cardiac and skeletal muscles. Conversely, in the fed state LPL in WAT is free from any inhibition and is able to hydrolyze TGs becoming available to be stored into adipocytes.

5. Role of ANGPTL3, 4, and 8 variants in hypertriglyceridemia and cardiovascular risk

Several studies have shown that ANGPTL3 LoF variants are associated with favorable modifications in TGs, LDL-C, and HDL-C plasma levels [39, 95–98]. Specifically, 35 non-synonymous sequence variants, including non-sense mutations and frameshift mutations, were identified in multi-ethnic individuals recruited in the Dallas Heart Study; all subjects carrying LoF mutations in ANGPTL3 were in the lowest quartile of TGs levels [39]. Variants of ANGPTL3 associated with low plasma TGs levels were also reported in subjects carrying the single-nucleotide polymorphism M259T, and in two individuals carrying nonsense mutations S17X and E129X [39, 95, 99]; remarkably, these subjects exhibited extremely low plasma TGs and LDL-C levels and almost no detectable ANGPTL3 in their plasma [99].

Frederick Dewey and colleagues recently reported that carriers of LoF mutations in ANGPTL3 have 27% lower TGs levels, and 9% lower LDL-C levels than non-carriers [2]. In 13,102 individuals with CAD, the presence of LoF mutations in ANGPTL3 was associated with a 41% lower risk of cardiovascular disease [2, 35, 100]. Similarly, Stitziel et al. observed in 1,493 patients with cardiovascular disease and 3,231 controls that, after adjustment for plasma TGs and LDL-C, the risk of myocardial infarction was reduced by

29% in individuals in the lowest tertile of ANGPTL3 concentrations compared with those in the highest tertile [35].

Consistently with the ANGPTL3–4-8 model, gene sequencing and pharmacological studies confirmed that LoF variants in ANGPTL4 induced a favorable lipid profile and reduced cardiovascular risk [1, 3, 39, 101, 102]. In 2007 Romeo and colleagues observed in a large population-based study a significant association between the inactivating variant E40K of ANGPTL4 and low TGs as well as high HDL-C plasma levels [102]. Successively, other investigators corroborated these findings, establishing that LoF mutations in human ANGPTL4 improve the circulating lipids profile [103–105]. Folsom et al. in 2008 found a significant association between the E40K variant and a lower risk of CAD [105]. More recently, Stitziel and colleagues conducted a cross-sectional study in 72,868 CAD patients and 120,770 controls in which the missense E40K and other inactivating variants were associated with a 35% reduction in TGs levels; the authors also observed that these LoF ANGPTL4 mutations were associated with higher levels of circulating HDL-C and a 53% lower risk of CAD compared with non-carriers [3, 30]; Dewey's group also identified 13 distinct mutations that were predicted to inactivate ANGPTL4, carried in heterozygosis in 75 participants (with a frequency of 1 in 572 participants) and these subjects exhibited the same reduction in plasma TGs levels observed by heterozygous E40K variant subjects [3]. Dewey's study also linked the ANGPTL4 E40K variant and other inactivating mutations in ANGPTL4 to increased levels of HDL-C [3, 30]. To explain these differences, Dewey and colleagues suggest a gene-dosage effect of the ANGPTL4 E40K variant on plasma TGs and HDL-C levels [3]. Yet, Dewey's work is in agreement with Stitziel's observations relative to CAD risk. Dewey and collaborators reported indeed the inverse relationship between CAD and E40K variant and other inactivating mutations of ANGPTL4 in 10,552 patients with CAD and 29,223 controls, after adjustment for age, sex, and ancestry. The presence of an inactivating mutation in ANGPTL4 lowered by 44% the risk of CAD compared to subjects non-carrier of inactivating mutations [3]. In 2018, Viktoria Gusarova and colleagues reported an association between ANGPTL4 inactivating variants and the improvement of glucose homeostasis and T2DM; subjects carriers of ANGPTL4 inactivating substitution E40K had a lower probability of T2DM (odds ratio 0.89, 95% confidence interval, $p = 6.3 \times 10^{-10}$), lower fasting glucose, and greater insulin sensitivity, compared to carriers of normal ANGPTL4 [106]. The Authors also observed that the ANGPTL4 substitution E40K was associated with lower fasting glucose in non-diabetic participants [106].

Population-based studies confirmed the association between ANGPTL8 LoF and the improvement of the circulating lipids profile [1, 57].

6. Conclusions

Since the discovery of its first member, ANGPTL3, in 1999, the ANGPTL3–4-8 triad has rapidly become one of the more studied regulatory factors of the circulating lipid profile. In the last two decades, the mechanisms underlying the ANGPTL3, 4, and 8 mediated regulation of TG and VLDL have been unraveled, and a considerable amount of in vivo and population-based studies have been conducted to investigate the metabolic effects of inactivating mutations in ANGPTL3, 4, 8 and their consequences in terms of cardiovascular

risk. One next challenge is to better clarify the role of ANGPTL3, 4, and 8 in glucose homeostasis, T1DM and T2DM, hypertension, and in general in the broad cluster of conditions that define metabolic syndrome. Another exciting challenge for scientists working on identifying new therapeutic strategies to treat metabolic disorders, is to create antibodies or ASOs targeting ANGPTL3, 4, and 8, in order to improve the lipidic profile in subjects with metabolic risk factors. Currently, some relevant results have been already obtained in this scenario, namely the first generation of antibodies against ANGPTL3 and ANGPTL8 (evinacumab and REGN3776) and ASOs targeting ANGPTL3. These innovative therapeutical approaches are at the beginning of their developing and further investigations are warranted in order to achieve safety levels for human application, but in the next years is expected that targeting ANGPTL3, 4, and 8 will be a promising therapeutic approach to treat metabolic diseases and eventually reduce cardiovascular risk.

7. Expert opinion

Elevated plasma TGs levels play an important role in the development of cardiovascular disorders and might be caused by impaired lipoprotein clearance [23]. Individuals with LoF mutations in ANGPTL3, similarly to rodents lacking ANGPTL3, have a favorable circulating lipid profile and reduced plasma levels of TGs, VLDL, and LDL-C [2, 107, 108]. Therefore, it is possible to speculate that subjects treated with therapeutical antagonists of ANGPTL3 are associated with a reduced risk of atherosclerosis [2, 107].

Consistent with these observations, the fully human monoclonal ANGPTL3-blocking antibody evinacumab has been shown to be able to bind with high affinity and specificity human, monkey, mouse and rat ANGPTL3, reversing its inhibitory activity on LPL both in vitro and in vivo [107], while in C57BL/6 mice, a dose-dependent reduction in TGs and LDL-C serum levels was detected after subcutaneous injections of evinacumab [107]. Moreover, the ANGPTL3-blocking antibody increased LPL activity in normolipidemic C57BL/6 mice as well as in dyslipidemic C57BL/6 and db/db mice; similar results have been obtained by evinacumab treatment in dyslipidemic cynomolgus monkeys [107]. In two randomized, double-blind, placebo-controlled, phase 1 studies, Dunbar and colleagues have demonstrated that the evinacumab is generally well-tolerated in patients with mixed dyslipidemia and borderline high TGs, and prompts substantial and sustained reductions in TGs and VLDL levels [23]. The reduction in TGs and other lipid subfractions by evinacumab is similar to the pan-hypolipidemia observed in individuals with LoF mutations in ANGPTL3 [23]. Evinacumab can induce a dose-dependent (placebo-adjusted) reduction in fasting TG levels, with a maximal decrease in lipid levels equal to −76.0% in TGs, −23.2% in LDL-C; and −18.4% in HDL-C [2]; besides, in dyslipidemic (ApoE* 3-Leiden) mice they demonstrated that evinacumab was associated with a significantly lower (−52%) total cholesterol level and a significantly lower (−84%) TG level compared with a control antibody [2]. Evinacumab was also associated with a significant decrease (−39%) in aortic root atherosclerotic lesion size and a marked decrease (−45%) in necrotic content in severe lesions compared with the control antibody; however, no difference was detected in macrophage content, collagen content, or smooth muscle cell area [2]. Strikimgly, the administration of evinacumab in adults with homozygous familial hypercholesterolemia resulted in substantial reductions in LDL-C levels [80].

Consistent with these results, in 2017 Graham and collaborators reported the cardiovascular and metabolic effects of ANGPTL3 Antisense Oligonucleotides (ASO), showing that targeting murine ANGPTL3 significantly retarded the progression of atherosclerosis and reduced the levels of atherogenic lipoproteins; specifically such approach induced dose-dependent reductions in levels of hepatic Angptl3 (both mRNA and protein), TGs, and LDL-C, as well as reductions in liver TG content; moreover, ANGPTL3 ASO treatment in mice significantly slowed down aortic atherosclerosis progression and increased insulin sensitivity [100]. When applied in the clinical scenario, the same strategy led to a reduction in atherogenic lipoprotein levels: 6 weeks of multiple-dose treatment reduced levels of ANGPTL3 protein, as well as levels of TGs, LDL-C, VLDL, apolipoprotein B, and apolipoprotein C-III, compared with placebo [100].

Hence, oligonucleotides targeting Angptl3 yielded remarkable results, significantly retarding the progression of atherosclerosis in mice, reducing levels of atherogenic lipoproteins both in mice and humans. This class of compounds is opening a possible application in a wide range of metabolic diseases, in which one or a few factors have a crucial regulation activity in lipid profile and/or glucose homeostasis. In light of these considerations, ASOs based strategies could be interesting as a new approach also for ANGPTL4 targeting, in order to overcome the cases of acute toxicity in mice reported by some investigators in ANGPTL4$^{-/-}$ mice or following antibody-based treatments [109, 110]. An alternative approach, based on a modified CRISPR-Cas9 platform (namely Base Editor 3, BE3), has been harnessed to permanently inhibit ANGPTL3 in vivo by introducing non-sense mutations within the murine ANGPTL3 gene; after injections of adenoviral vectors expressing BE3-ANGPTL3 in C57BL/6 wild-type mice and hyperlipidemic LDLR$^{-/-}$ mice, TGs and total cholesterol levels were halved compared with control mice [111].

The inhibition of ANGPTL4 has been shown to reduce levels of circulating TGs in ANGPTL4-deficient dyslipidemic mice (ApoE$^{-/-}$ANGPTL4$^{-/-}$), associated with better lipid metabolism, and a significant reduction of aortic atherosclerotic lesions compared with control mice (ApoE$^{-/-}$ANGPTL4$^{+/+}$) [112]. Furthermore, on high-fat diet, ANGPTL4$^{-/-}$ mice exhibited reduced circulating TGs and cholesterol levels associated with 31% lower non-fasted blood glucose, improved glucose tolerance and insulin sensitivity [106]; instead, on chow diets, glucose levels and glucose tolerance were not significantly different between ANGPTL4$^{-/-}$ and ANGPTL4$^{+/+}$ littermates [106].

Consistent with the results in dyslipidemic ANGPTL4$^{-/-}$ mice, the ANGPTL4-neutralizing fully human monoclonal antibody (REGN1001) has been shown to reduce plasma TGs levels in ANGPTL4-deficient dyslipidemic mice as well as in obese cynomolgus monkeys [3]. However, different investigators have reported in ANGPTL4-deficient and ANGPTL4 antibody-treated mice cases of acute toxicity due to abdominal lymphadenopathy, most likely attributable to granulomatous lipid accumulation, [109, 110]. Nonetheless, Gusarova and colleagues did not observe cases of toxicity or precarious health conditions in ANGPTL4-deficient mice after 9 weeks on a high-fat diet [106], and no cases of toxicity in E40K homozygous subjects have been reported until now [3, 106].

Even if the potential benefits of ANGPTL4 blockade turn out to be limited by the risk of lymphadenopathy, similar therapeutic benefits may still be achieved by blocking ANGPTL4-related proteins, namely ANGPTL8 and ANGPTL3, neither of which appears to be associated with lymphadenopathy [3].

Of note, recent data suggest a role for ANGPTL5 – mainly expressed in adipose tissue and heart – also in lipid metabolism [39, 113], although the molecular mechanisms underlying such association have not been clarified hitherto.

Since ANGPTL8 plays a crucial role in the inhibition of LPL via enhancing the ANGPTL3 activity, we can speculate that a strategy to reducing the LPL enzyme activity in subjects with unfavorable lipids profile is to block ANGPTL8. This assumption is confirmed by preclinical studies, in which the hepatic overexpression of ANGPTL8 in mice is associated with hypertriglyceridemia, whereas genetic inactivation of ANGPTL8 reduces plasma TGs [1, 81]. Moreover, ANGPTL8$^{-/-}$ mice exhibit reduced body weight and fat mass if compared with control mice [81]. On the basis of this previous evidence, Gusarova and collaborators studied the inactivation of ANGPTL8 using a human monoclonal antibody (REGN3776); this antibody shows high and comparable affinity to ANGPTL8 from monkeys and humans, however, REGN3776 does not bind mouse ANGPTL8. Since it was not available a specific antibody to bind ANGPTL8 in mice, the model was humanized (ANGPTL8$^{hum/hum}$ mice) [57]; the investigators observed that a single administration of REGN3776 to chow-fed ANGPTL8$^{hum/hum}$ mice significantly reduced circulating TGs compared with control antibody treatment, but it did not affect total cholesterol. The reduction in TGs was sustained for 14 days and was associated with a marked increase in plasma levels of ANGPTL8 [57]. REGN3776 long term treatment (16 weeks) in ANGPTL8$^{hum/hum}$ mice led to a sustained and significant reduction in postprandial circulating TGs and in body fat, compared with control mice. Of note, REGN3776 reduced the body weight but did not alter TG content in the liver, heart, muscle, and BAT, and WAT; REGN3776 was also tested in monkeys, showing a sustained dose-dependent reduction in circulating TGs up to 65% within 1 day following antibody administration, compared to control animals; moreover, the inhibition of ANGPTL8 increased the levels of HDL-C by 30% at all doses but did not change LDL-C levels [57].

In line with these experiments, Vatner et. al reported that high-fat-fed Sprague Dawley rats and C57BL/6 mice treated with a second-generation of 2′-O-methoxyethyl ASO against Angptl8 displayed a significant improvement of glucose tolerance. Moreover, high-fat-fed rats treated with Angptl8 ASO for 3 weeks prevented hepatic steatosis, PKCε activation, hepatic insulin resistance, and increased postprandial triacylglycerol uptake in WAT, strongly suggesting that targeting ANGPTL8 with ASO can prevent, at least in rodents, lipid-induced insulin resistance [114]. Taken together, these results are remarkable and can open the way to new strategies to treat dyslipidemia that could have noteworthy effects in the prevention of cardiovascular disorders.

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ABBREVIATIONS

AMPK	5’ adenosine monophosphate (AMP)-activated protein kinase
ANGPTL	Angiopoietin-like
Apo	Apolipoprotein
ASO	antisense oligonucleotide
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CAD	Coronary Artery Disease
ChER	Carbohydrate Responsive Element
ChERBP	Carbohydrate responsive element-binding protein
GWAS	Genome Wide Association Study
HDL	High-Density Lipoprotein
HDL-C	HDL Cholesterol
HepG2	human liver hepatocellular lines
HL	hepatic lipase
HNF-1α	Hepatocyte Nuclear Factor 1α
HOMA	homeostasis model assessment
IDR	Intrinsically Disordered Region
LDL	Low-Density Lipoprotein
LDL-C	LDL Cholesterol
LoF	Loss-of-function
LPL	Lipoprotein lipase
LXRs	Liver X Receptors
SREBP-1c	Sterol Regulatory Element-Binding Protein-1c
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGs	Triglycerides
VLDL	Very-Low-Density Lipoprotein

WAT White Adipose Tissue

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Article Highlights

- ANGPTL3, ANGPTL4, and ANGPTL8 (the 3–4-8 triad) are largely studied for their crucial role as negative regulators of LPL activity.
- ANGPTL8 functions as a metabolic switch by forming complexes with ANGPTL3 or ANGPTL4.
- Subjects with LoF mutations in ANGPTL3 have reduced plasma level of TGs, VLDL, and LDL-C.
- The missense E40K variant in ANGPTL4 protein associates with decreased levels of TGs and increased levels of HDL-C.
- Monoclonal antibodies targeting ANGPTL3, ANGPTL4, and ANGPTL8 are effective in reducing TG levels
- Monoclonal antibodies and antisense oligonucleotides that target ANGPTL3, 4, and 8 are potentially an efficient therapeutic strategy for hypertriglyceridemia and cardiovascular risk reduction, especially in patients with limited treatment options. These innovative therapeutical approaches are at an embryonic stage in development and hence further investigations are necessary for eventual use in humans.

Table 1.

Angiopoietin-like proteins.

ANGPTL	Other common names	Chromosome (human)	Chromosome (mouse)	Main tissue expression	References
1	Angioarrestin, ARP1, ANG3, AngY, ANGPT3, UNQ162, dJ595C2.2, 2810039D03Rik	1	1	Liver, heart, skeletal muscle, connective tissue and cartilage, kidney, and vessel-rich endocrine organs (adrenal glands, thyroid, and pituitary gland), uterus and gastrointestinal tract	[79, 115, 116]
2	ARP2, AI593246, AW260363, HARP	9	2	Heart, stomach, adipose tissue, skeletal muscle, uterus	[117, 118]
3	ANL3, ANG-5, FHBL2, ANGPT5	1	4	Liver	[23, 70]
4	ARP4, PGAR, HFARP, FIAF	19	7	Liver, adipose tissue, skeletal muscle, intestine, heart, brain	[64, 119, 120]
5	ANGL5_HUMAN, A_14_P125422, NP835228.1	11	-	Heart	[121, 122]
6	AGF, ARP5, ARP3	19	9	Liver	[123–125]
7	AngX, dJ647M16.1, CDT6, RP4–647M16.2	1	4	Eye (trabecular meshwork, cornea), neural tissues, uterine endometrial	[126, 127]
8	Betatrophin, lipasin, c19orf80, PRO1185, PVPA599, Gm6484, RIFL, TD26	19	9	Liver, adipose tissue	[1, 56, 128]

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