



T.C.  
EGE UNIVERSITY  
Institute of Health Sciences



**INVESTIGATION OF ENDOCRINE DISRUPTING  
AND OBESOGENIC EFFECTS OF SEVERAL  
PHARMACEUTICALS THAT ARE EXPOSED IN  
THE EARLY STAGES OF LIFE**

**PhD Thesis**

Deniz BOZDAĞ

Department of Pharmaceutical Toxicology

İzmir  
2024

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## **Preface**

In 2019, I graduated from the Faculty of Pharmacy, at Ege University. During the last year of my study, I completed my finishing thesis at the Department of Pharmaceutical Toxicology with my supervisor, Prof. Dr. Hande Gürer Orhan. Afterwards, it became clear to me that I wanted to advance in the field of toxicology, by pursuing a doctorate degree which was an important step in fulfilling my dream of becoming a scientist.

As a first-year PhD student, I became involved in the field of Endocrine Toxicology, focusing on the obesogenic and endocrine-disrupting properties of two antidepressants. In the third year of my PhD, we conducted a collaborative project with Ass. Prof. Dr. Jorke Kamstra at the Institute of Risk Assessment Sciences, Utrecht University. Further into my studies at Utrecht University, we discovered the unexpected effects of these pharmaceuticals on human mesenchymal stem cells, which ultimately led my research to progress in a different way and allowed me to appreciate unpredictability as one of the beauties of doing science, as the most valuable lessons are learned especially when things don't go as planned.

Pursuing a PhD was challenging to say the least, and yet it was one of the best decisions I have ever made. I learned a lot in this process under the guidance of my supervisors, who helped me grow both as a scientist and as a person.

I am very proud to present to you my doctoral thesis, one of the most precious outcomes of my hard work and efforts for the past few years.

İzmir, 23.06.2024

Deniz Bozdağ



## Geniř Trke zet

### Yařamın Erken Dnemlerinde Maruz Kalınan Bazı İlaların Endokrin Bozucu ve Obezojenik Etkilerinin Arařtırılması

Dnya Saęlık rgt (DS), ařırı kilo ve obeziteyi, “*vcutta anormal ve saęlıęı tehdit eden dzeyde yaę birikimi*” olarak tanımlamaktadır. DS’ye gre obezite dnyada ilk 10 saęlık riski arasında yer almakta ve dnya genelinde giderek artan bir kitleyi etkilemektedir. DS 2016 verilerine gre, dnya genelindeki yetiřkinlerin % 39’u (1.9 milyar kiři) ařırı kilolu ve % 13’ (650 milyon kiři) obez olarak sınıflandırılmaktadır. 2019 verilerine gre, 5 yařın altındaki ocukların 38 milyondan fazlası ařırı kilolu veya obez olarak sınıflandırılmaktadır. Obezite prevalansı, 1975-2016 yılları arasında dnya genelinde neredeyse  kat artarak, gnmzde byk bir endiře kaynaęı olarak grlmektedir (WHO 2024).

Obezite tek bařına bir hastalık olarak deęerlendirilmekle birlikte, tip 2 diyabet ve alkole baęımlı olmayan karacięer yaęlanması gibi metabolik bozukluklar, kardiyovaskler hastalıklar, bazı kanser trleri ile sıklıkla iliřkilendirilmektedir (Casals-Casas and Desvergne 2011). Obezitenin yetiřkinler arasında nemli bir saęlık sorunu olmasının yanı sıra, son yıllarda daha erken yařlarda obezite ile birlikte tip 2 diyabet vakalarında dramatik bir artıř grlmektedir. Bu durum, ocukluk dnemi obezitesinin de ciddi bir saęlık problemi haline geldięini gstermektedir ve ocukluk dneminde obezitenin nlenmesi, ilerleyen yařlarda obezite ve metabolik hastalıkların geliřimini kontrol etmek iin nemli bir adımdır (Darbre 2017).

Obezitenin temel nedeni bireyde alınan ve harcanan kaloriler arasındaki dengesizlik ile birlikte genetik yatkınlık olarak kabul edilmektedir (WHO 2024). Fazla kalorili gıdaların ařırı tketimi ve fiziksel aktivitenin azalması sonucu oluřan enerji dengesizlięi obezite geliřiminde nemli role sahip olsa da, bu faktrler son yıllarda grlen dramatik artıřı aıklamada yetersiz kalmakta ve obezite, genetik yapı, yařam tarzı ve evresel faktrlerin geliřimine katkıda bulunduęu karmařık bir endokrin-iliřkili hastalık olarak tanımlanmaktadır (Casals-Casas and Desvergne 2011). Fazla beslenme ve egzersiz eksiklięi gibi faktrlere ek olarak, kimyasallara artan dzeyde maruz kalmanın da obezite vakalarındaki hızlı artıřa katkıda bulunduęu dřnlmektedir. Kimyasalların endokrin sistem fonksiyonlarıyla etkileřerek istenmeyen etkilere yol atıęı 1990’lı yılların bařından beri bilinmektedir. Biyolojik

ve fizyolojik fonksiyonlardaki kritik rolü nedeniyle endokrin sistemde oluşacak herhangi bir bozukluk, üreme ve metabolizma işlevinde bozukluk, bağışıklık ve sinir sistemi fonksiyon bozuklukları ve kanser riskinde artış gibi istenmeyen etkilerle sonuçlanmaktadır (Gore et al. 2014). Endokrin bozucu kimyasallar (EB) “*endokrin sistem fonksiyonlarını değiştirerek, maruz kalan eksiksiz/bozulmamış (intact) organizma, gelecek nesiller veya (sub)popülasyonları üzerinde olumsuz sağlık etkilerine neden olan kimyasal madde ya da karışımlar*” olarak tanımlanmıştır (A. Bergman et al. 2012). EB’ler, normal endokrin fonksiyonlar için gerekli olan, hormonların sentezini ya da yıkımını değiştirerek, hormon reseptörleri ile etkileşerek (agonist/antagonist etki) ya da reseptör ekspresyonu üzerine etki ederek endokrin sistem fonksiyonunda değişikliklere neden olmaktadır (Gore et al. 2014).

2000’li yılların başından itibaren kimyasalların obezite gelişimindeki rolü artan sayıda araştırmanın konusu olmuş, bu çalışmalardan elde edilen verilere göre çeşitli mekanizmalarla adipoz doku gelişimini ve fonksiyonunu etkileyerek lipid birikimini artıran EB’ler “obezojen” olarak adlandırılmıştır (Grün et al. 2006). Obezojenler, hayvan modelleriyle kilo alımına neden olduğu gösterilen ve endokrin sistemin kilo alımını kontrol eden bileşenleri ile (adipoz doku, beyin, iskelet kası, karaciğer, pankreas ve gastrointestinal kanal) etkileşen kimyasallar olarak tanımlanır (A. Bergman et al. 2012). Obezojenler adiposit sayısını/boyutunu artırarak, adipoz doku gelişimini kontrol eden endokrin yollarla etkileşerek, iştah, tokluk ve gıda tercihlerini düzenleyen hormonlarla etkileşerek, pankreas, adipoz doku, karaciğer, gastrointestinal sistem, beyin ve kaslar gibi endokrin (ve endokrin ilişkili) dokularda insülin duyarlılığını ve lipid metabolizmasını değiştirerek obezite gelişimini indükleyici etki göstermektedir. Obezojenlere maruz kalım ile kilo artışı ve obezite arasındaki ilişki *in vitro*, *in vivo* ve epidemiyolojik çalışmalarla kanıtlanmış, prenatal maruz kalımın ise yaşamın ilerleyen dönemlerinde obeziteye neden olabileceği öne sürülmüştür. Ancak, obezojenlerin etki gösterdiği çeşitli mekanizmalar ve obezite gelişimindeki rolü hakkındaki bilgi sınırlıdır ve ileri çalışmalarla aydınlatılması gerekmektedir.

Yağ dokusu temel olarak olgun adiposit hücrelerinden oluşur ve bu hücreler obezite ile metabolik sendrom gelişiminde anahtar role sahiptir. Adiposit progenitor hücrelerin çeşitli nükleer reseptörlerin etkisi ile olgun adiposit hücrelerini oluşturması “adipojenenez” olarak tanımlanmaktadır (Janesick and Blumberg 2012). Moleküler

düzeyde obezojenlerin çoğu adipojeniz sürecinde yol alan nükleer reseptör ailesine dahil olan transkripsiyon faktörlerini etkileyerek obezojenik etki gösterir. *PPAR $\gamma$* , adipojenizde rol alan transkripsiyon faktörleri arasında en kritik role sahiptir. Obezojenlerin çoğu, *PPAR $\gamma$*  aktivasyonu ile adiposit farklılaşmasını ve adipojenik gen ekspresyonunu artırarak insanlarda ve hayvanlarda obeziteye neden olmaktadır.

*PPAR $\gamma$*  aktivasyonu, obezojenlerin etkilerini gösterdiği birincil mekanizma olmakla birlikte, adiposit farklılaşması üzerinde etkili olan başka reseptör sistemleri de vardır (Casals-Casas and Desvergne 2011). Hayvan çalışmaları, östrojenik etkili obezojenlere prenatal maruz kalımın, yavrularda obezite gelişimini indüklediği ve bu etkinin cinsiyete göre değiştiğini göstermiştir. Bu etkilerin östrojen reseptörü (ER) aracılığıyla gerçekleştiği düşünülmektedir (Retha R. Newbold et al. 2007). Arilhidrokarbon reseptörü (AhR), ligand bağlanması ile aktive olan bir transkripsiyon faktörüdür. AhR'nin *PPAR $\gamma$*  ekspresyonunu inhibe ederek adipojenizi dolaylı olarak etkilediği bilinmektedir. Obezojenik aktiviteye sahip bazı obezojenlerin bu mekanizmayla adipojenizi indüklediği gösterilmiştir (Casals-Casas and Desvergne 2011).

Obezojenlerin çoğu lipofilik yapılarından ötürü adipoz dokuda birikmekte ve plasentadan fetüse geçerek adipoz doku gelişimini değiştirmektedir (Darbre 2017). Son yıllarda özellikle kimyasallara prenatal maruz kalım ile çocuklarda ileri dönemde obezite gelişimi arasındaki ilişkiyi araştıran çalışmaların sayısı artmıştır (Silver and Meeker 2015). Bu çalışmalarda yaşamın erken evrelerinde obezojenlere maruz kalım ile ileri dönemlerde obezite gelişimi arasında pozitif bir korelasyon gösterilmiştir (Diamanti-Kandarakis et al. 2009). Gelişimin erken dönemlerinde obezojenlere maruz kalımın adiposit oluşumunu değiştirerek enerji homeostazının bozulmasına ve ilerleyen yaşlarda obezite ve diyabet gibi metabolik hastalıkların gelişimine yol açabileceği düşünülmektedir. Özellikle organojenez döneminde metabolik süreçler ile etkileşen kimyasallara maruz kalımın çocukluk dönemi obezite gelişiminde önemli rol oynadığı literatürde bildirilmektedir (La Merrill and Birnbaum 2011).

Avrupa Birliği, 2020 yılında kimyasalların metabolik süreçler ve adipoz doku fonksiyonu üzerindeki etkilerinin taranması ve test edilmesinde kullanılacak *in silico*, *in vitro* ve *in vivo* test yöntemlerinin geliştirilmesi için geniş kapsamlı bir çalışma başlatmıştır. Bu çalışmanın amacı, obezojenik etkili kimyasalların belirlenmesi için valide edilmiş test modellerinin geliştirilerek kimyasalların risk değerlendirmesine

katkıda bulunmaktır (Legler et al. 2020). Günümüzde organotinler, bisfenollar, ftalatlar gibi birçok endüstriyel/sentetik kimyasalın endokrin bozucu ve obezogenik etki potansiyelleri fazla sayıda *in vitro*, *in vivo* ve insan çalışmalarıyla net bir şekilde ortaya konmuştur. Söz konusu kimyasalların büyük çoğunluğu plasentadan fetüse geçerek yaşamın ileri evrelerinde çeşitli hastalıkların görülme riskini artırmaktadır. Bununla birlikte yarar/zarar dengesi gözetilerek gebelik sürecinde reçete edilen birçok ilacın, anne karnında fetal maruz kalıma bağlı olarak yaşamın ileri evrelerinde ortaya çıkabilecek endokrin bozucu ve obezogenik etki potansiyelleri hakkında literatürde yeterli veri bulunmamaktadır.

Selektif serotonin geri alım inhibitörü (SSRI) grubu antidepresanlar, dünya genelinde en sık reçete edilen ilaçlar arasındadır. Amerikan Gıda ve İlaç Dairesi'nin (FDA) eski gebelik sınıflandırmasında C kategorisinde yer alan ve gebelik sırasında da yarar/zarar dengesi gözetilerek reçelenen SSRI'ların maternal ve neonatal güvenliği gittikçe artan bir endişe kaynağıdır. SSRI'ların üreme sistemi üzerindeki istenmeyen etkileri literatürde birçok araştırmanın konusu olmuştur. Epidemiyolojik çalışmalarda SSRI ilaçlardan birini kullananların % 30-60'ında cinsel fonksiyon bozuklukları görüldüğü bildirilmiştir (Gregorian Jr et al. 2002). Bir başka epidemiyolojik çalışmada, SSRI grubu ilaçları kullanan yetişkin erkeklerde fertilite, testosteron, luteinize edici hormon (LH), folikül stimüle edici hormon (FSH) düzeylerinde azalma, prolaktin düzeyinde artış gibi hormonal değişiklikler bildirilmiştir (Safarinejad 2008). Bu bulgular, *in vitro* çalışmalarla desteklenmiştir, iki farklı mikrozom temelli *in vitro* aktivite deneyinde SSRI grubu ilaçlardan sitalopram (CIT) ve sertralinin (SER) aromataz enzimini inhibe ederek östrojen düzeyini azalttıkları gösterilmiştir (Jacobsen et al. 2015). Erkek sıçanlarla gerçekleştirilen *in vivo* çalışmalarda SER'in testis ve adrenal bezlerde steroid hormon üretimini etkilediği (Munkboel et al. 2018), sperm sayı ve motilitesinde azalmaya neden olduğu gösterilmiştir (Atli et al. 2017). Ancak literatürde CIT'in EB etkilerini araştıran *in vivo* herhangi bir çalışmaya rastlanmamıştır.

Bununla birlikte, son yıllarda antidepresan kullanımı ile kilo artışı ve obezite arasındaki ilişkiyi ortaya koyan çalışmaların sayısı artmaktadır. Epidemiyolojik çalışmalarda uzun süreli SSRI tedavisi, hastalarda vücut ağırlığında önemli bir artış ile ilişkilendirilmiştir (Arterburn et al. 2016; Blumenthal et al. 2014; Gafoor, Booth, and Gulliford 2018). SSRI'lar arasında sıklıkla reçete edilen CIT ve SER'in ise kilo

alımına yol açma riskinin yüksek olduğu ortaya konmuştur (Gill et al. 2020). Ancak literatürde CIT ve SER'in potansiyel obezogenik etkileri ve bu etkilerin olası mekanizmalarının araştırıldığı *in vitro* veya *in vivo* bir çalışmaya rastlanmamıştır.

Bu bilgilerden hareketle, hipotezimiz CIT ve SER'in obezogenik potansiyele sahip olduğu, bu etkilerin endokrin-ilişkili mekanizmalarla gerçekleşebileceği ve bununla birlikte gebelikte yarar/zarar oranı gözetilerek reçete edilen CIT ve SER'e anne karnında maruz kalan kişilerde ileri yaşlarda obezogenik etkilerin ortaya çıkabileceği yönündedir. Bu tez çalışması kapsamında, CIT ve SER'in potansiyel obezogenik etkileri ve bu etkilerin olası mekanizmalarının *in vitro* yöntemlerle araştırılması ile yukarıda belirtilen hipotezlerimizin test edilmesi hedeflenmiştir.

Bu hedefler doğrultusunda, ilk olarak ilaçların obezogenik etkileri *in vitro* adipojenez yöntemiyle, insan multipotent mezenkimal kök hücreleri (MKH'ler) ve 3T3-L1 fare preadiposit hücreleri kullanılarak araştırılmıştır. *In vitro* adipojenez yöntemi, kimyasalların obezogenik etkisinin belirlenmesinde sıklıkla kullanılan yöntemler arasındadır. Adiposit progenitor (3T3-L1) veya multipotent kök hücreler (MKH'ler) gibi hücre hatlarının kullanıldığı bu yöntemde, olgun adipositlere farklılaşma hücre içi lipit birikimi kantitatif olarak ölçülerek değerlendirilir. *In vitro* modeller, basitlikleri, *in vivo* yöntemlere kıyasla maliyetlerinin uygunluğu ve yüksek verimli taramaya (high-throughput screening) olanak vermeleri nedeniyle tercih edilmektedir. Yaygın olarak kullanılan 3T3-L1 hücre hattı iyi karakterize edilmiş olsa da, sonuçların farklı kaynaklardan elde edilen hücreler arasında farklılık gösterdiği ve *PPAR $\gamma$*  aktivasyonundan farklı bir mekanizma aracılığıyla etki gösteren obezogenlerin belirlenmesinde yetersiz kaldığı bildirilmektedir (Kassotis et al. 2021). Bunun yanı sıra, 3T3-L1 hücre hattının adiposit soyuna bağlı olması nedeniyle bu yöntemden MKH'lere kıyasla daha sınırlı bilgi elde edilebilmektedir. MKH'ler, multipotent kökenlerinden ötürü beyaz, bej ve kahverengi adipositlerin yanı sıra osteoblastlar ve kondroblastlar gibi çeşitli hücre tiplerine farklılaşmak üzere programlanabilmektedir. Bu özellikleri, adiposit farklılaşmasının yanı sıra hücrelerin adiposit soyuna bağlılığının değerlendirilmesine olanak vermektedir (Kassotis et al. 2022). Buna ek olarak, çoğu çalışmada iki boyutlu (2D) monolayer kültürler kullanılmaktadır. Ancak 2D farklılaştırılan adipositler *in vivo* adipoz dokuyla kıyaslandığında morfoloji, boyut ve gen ekspresyonunda önemli farklılıklar gözlenmiştir. Son çalışmalar, üç boyutlu (3D) sistemlerin iyileştirilmiş adiposit farklılaşması ve gen ekspresyonu ile *in vivo*

koşulları daha iyi temsil ettiğini göstermektedir (Klingelhutz et al. 2018; Muller et al. 2019; Shen et al. 2021). Bu nedenle son yıllarda adipojenik etkilerin araştırılmasında 3D adipojenez modelleri giderek daha fazla tercih edilmektedir.

Adipojenez deneylerini takiben, olası etki mekanizmaları adipojenik gen ve protein ekspresyonu araştırılarak aydınlatılmıştır. Endokrin yollarla ilişkili mekanizmalar, *in vitro* reseptör bağlanma ve transaktivasyon yöntemiyle ER, AR, AhR ve peroksizom proliferatör ile aktive edilen reseptör (PPAR) eksprese eden haberci hücre hatlarıyla, ek olarak hormon-bağımlı proliferasyon ve aromataz enzim aktivitesi ölçümü yöntemleriyle araştırılmıştır.

### ***Mezenkimal kök hücrelerle (MKH) gerçekleştirilen in vitro adipojenez deneyi***

Multipotent insan MKH'leri 15 % FBS, 1 % Pen-Strep içeren MEM- $\alpha$  medium (hücre ortamı) ile sürdürülmüş, *in vitro* adipojenez deneyleri pasaj 6'da gerçekleştirilmiştir.

#### ***3D model***

MKH'ler 96 kuyucuklu ULA (ultra low attachment) plakalara ekilmiş ve plakalar 150 g'de 2 dakika boyunca santrifüj edilmiştir (-2. gün). Hücre ekiminden 2 gün sonra (0. gün) sferoid oluşumu mikroskop altında belirlenmiş ve adipojenez, sferoidlere hasar vermemek için hücre ortamı hacminin yarısı farklılaşma mediumu (test maddeleri, 0.5 mM IBMX, 0.1  $\mu$ M Dex ve 5  $\mu$ g/mL insulin içeren hücre ortamı) ile değiştirilerek indüklenmiştir. Farklılaşma mediumu aynı şekilde (hacmin yarısı değiştirilerek) 3-4 günde bir tazelenerek deney 14 gün boyunca sürdürülmüştür. 0.5  $\mu$ M ROSI deneylerde pozitif kontrol, 0.1 % DMSO ise taşıyıcı kontrol olarak kullanılmıştır.

14. günde sferoidler boyanarak (lipit damlacıkları: Nile Red, hücre çekirdeği: Hoechst 33342) floresans mikroskopi görüntüleri alınmış (CellInsight™ CX5 High-Content Screening (HCS) Platform, Thermo Scientific) ve CellProfiler yazılımı (v4.2.4) kullanılarak analiz edilmiştir (Stirling et al. 2021). Floresans şiddetindeki artış, lipit birikimi ve adipojenezin kantitatif olarak belirlenmesinde kullanılmıştır.

DMSO ve ROSI grubuna ait sferoidlerin morfolojileri Hematoksilen & Eozin (H&E) boyama ile histolojik analizlerle, ve konfokal mikroskopi ile belirlenmiştir. H&E boyama ve konfokal mikroskopi, sferoidlerde herhangi bir nekroz belirtisi olmaksızın lipit damlacıklarının homojen bir şekilde dağıldığını göstermiştir. ROSI uygulaması,

lipit birikimi ve sferoid boyutunu artırmış, bu etkiyi hücre proliferasyonunda belirgin bir artışa neden olmaksızın göstermiştir. 2D monolayer adipositlerin floresans mikroskopi görüntüleriyle karşılaştırıldığında, 3D sferoidlerde, *in vivo* yağ dokusuna daha benzer bir fenotipte, az sayıda ve daha büyük lipit damlacıklarının olduğu görülmüştür. Bu fenotip, özellikle ROSI uygulaması ile belirgin şekilde ortaya çıkmıştır. Transkriptomik analizler 3D ve 2D modelde ROSI uygulaması sonucu metabolik yolların benzer şekilde etkilendiğini göstermiş, test edilen referans obezojenler ile iki modelde de adiposit farklılaşmasının benzer şekilde indüklendiği gösterilmiştir. Özetle, 3D modelin SSRI'ların obezojenik etkilerinin araştırılmasında uygulanabilirliği kanıtlanmıştır.

SSRI'lar 3D MKH'lerde lipit birikimini indükleyerek adipojenik etki göstermiş ve bu indüksiyon referans bileşik ROSI ile görülen seviyede gözlenmiştir. Benchmark doz (BMD) modelleme ile, bu etkinin literatürde belirtilen kararlı-durum plazma konsantrasyonları (SSC) aralığında olduğu ortaya konmuştur (CIT: 0.12–0.92  $\mu$ M, SER: 0.065–0.65  $\mu$ M) (Baumann 1996; De Vane, Liston, and Markowitz 2002).

### **2D model**

MKH'ler 24 kuyucuklu plakalara ekilmiş (-4. gün), dört gün sonra (0. gün) hücrelerde adipojenez farklılaşma mediumu ile indüklenmiştir. Deney, 3-4 gün aralıklarla test maddelerini içeren farklılaşma ortamı tazelenerek 14 gün boyunca sürdürülmüştür. Yöntemin kısmi validasyonu referans bileşik ROSI ile gerçekleştirilmiş, 0.5  $\mu$ M ROSI deneylerde pozitif kontrol, 0.1 % DMSO ise taşıyıcı kontrol olarak kullanılmıştır.

14 günlük farklılaşma sürecinin sonunda adipositler boyanarak (lipit damlacıkları: Nile Red, hücre çekirdeği: Hoechst 33342) floresans şiddeti çoklu plaka okuyucuyla ölçülmüştür. SER ve CIT ile yapılan deneylerde adiposit farklılaşmasında artış ve lipit birikimi ışık mikroskobu ile görüntülenmiş fakat floresans plaka okuyucunun bu etkileri saptamak için yeterince hassas bir yöntem olmadığı görülmüştür. Bu nedenle adipojenezin kantitatif olarak değerlendirilmesi için akış sitometrisi yöntemi kullanılmış, bu yöntemle mikroskop altında gözlemlenen etki kantitatif olarak ölçülebilmektedir.

### ***RNA dizilimi (RNA-sequencing) analizi***

Adipojenez deneylerini takiben, adipojenik etkinin altında yatan mekanizmanın daha kapsamlı olarak araştırılması için MKH'lerde RNA dizilimi analiz edilmiştir. Test maddeleri, 3D modelde, etki gözlenmeyen en düşük konsantrasyon (NOEC) ve etki gözlenen en düşük konsantrasyonda (LOEC) test edilmiş (SER 0.1 ve 1  $\mu$ M, CIT 1 ve 10  $\mu$ M), 0.1  $\mu$ M ROSI referans bileşik olarak kullanılmıştır. 3D deneyler protokole uygun şekilde gerçekleştirilmiş, 14. günde sferoidler toplanarak birleştirilmiş (test edilen her grup için n=8-10 sferoid), RNA örnekleri izole edilerek saflaştırılmış ve RNA-seq analizi gerçekleştirilmiştir. RNA-seq analizi için birbirinden bağımsız üç deney gerçekleştirilmiş, bu şekilde her test grubu için üç bağımsız örneğin analizi sağlanmıştır.

RNA-seq analizi için ilk olarak kalite kontrol analizleri gerçekleştirilmiş ve tüm örneklerin analize uygun kalitede olduğu saptanmıştır (Dobin et al. 2013). Bağımsız örnekler arasındaki farklılık PCA (principal component analysis plot) grafiği ile ortaya konmuştur. Bu grafikte test gruplarına ait örnekler ve kontrol grubu (DMSO) örnekleri birbirine yakın yerleşim gösterirken, referans bileşiği (ROSI) grubuna ait örneklerin, kontrol grubundan uzakta kümелendiği gözlenmiştir. Test maddelesi uygulaması sonucu ekspresyonu anlamlı olarak değişen genler Deseq2 yöntemiyle (Love, Huber, and Anders 2014) belirlenmiştir. Etkilenen biyolojik yollar bir web bazlı analiz uygulaması (WebGestalt) ve KEGG (Kyoto Encyclopedia of Genes and Genomes) veri bankası kullanılarak belirlenmiştir (Liao et al. 2019).

RNA-seq analizi sonucu referans bileşik ROSI ile adipojenez ile ilişkili yolların upregüle olduğu, fakat SER ve CIT uygulamasının tamamen farklı bir profile yol açarak metabolik yolların downregülasyonuna neden olduğu görülmüştür. Buna ek olarak beklenmedik şekilde lizozom ve fosfolipitlerle ilişkili yolların SER ve CIT uygulaması sonucu upregüle olduğu ve bu etkinin doza bağımlı olduğu gözlenmiştir.

MKH'lerde adipojenik gen ekspresyonu QPCR analiziyle araştırılarak RNA-seq analizi bulguları doğrulanmıştır. QPCR analiziyle adipojenezin regülatörleri (*PPAR $\gamma$* , *CEBPA*), PPAR sinyal yolağında bulunan hedef genler (*FABP4*, *ADIPOQ*) ve glukoz homeostazı ve insülin sinyalizasyonunda rol alan genler (*INSR*, *IGF1R*) incelenmiştir. SSRI'lar pozitif kontrol ROSI'ye kıyasla farklı bir ekspresyon profili göstererek



adipojenik genlerin downregülasyonuna yol açmıştır. QPCR analizi sonuçları, RNA-seq bulgularını doğrular niteliktedir.

### ***MKH'lerde lizozom/fosfolipitlerin analizi***

RNA-seq analizi ışığında, ilaçların adipojenez sırasında farklı lipit profilleri üzerindeki etkileri Nile Red boyama ile 2D MKH'lerde araştırılmıştır. Ek olarak ilaçların adipojenez sırasında lizozomları indükleyici etkisi lizozom-spesifik boyamayla (LysoTracker Red) belirlenmiştir.

MKH'ler 15 % FBS, 1 % Pen-Strep içeren MEM- $\alpha$  medium ile sürdürülmüş, deneyler pasaj 6'da, 2D protokolüne göre gerçekleştirilmiş, 14. günde olgun adipositler boyanarak (lipit damlacıkları: Nile Red, hücre çekirdeği: Hoechst 33342) floresans plaka okuyucu (nötral lipitler: Ex/Em 485/590 nm, fosfolipitler: Ex/Em 585/645 nm) veya akış sitometrisi kullanılarak analiz edilmiştir. 0.5  $\mu$ M ROSI ve antiaritmik ajan Amiodaron (AMIO, 10  $\mu$ M) sırasıyla nötral ve fosfolipit indüksiyonu için referans bileşik olarak kullanılmıştır.

Nile Red'in farklı kanallardaki (FITC ve Texas Red) floresans mikroskopi görüntüleriyle, lipitler arasındaki ayrım ortaya konmuş, yöntemin nötral ve fosfolipitlerin analizinde uygulanabilirliği kanıtlanmıştır. CIT ve SER MKH'lerde adipojenez sırasında nötral lipit birikimini artırmıştır. Ancak, her iki SSRI ile fosfolipit kanalında daha güçlü bir indüksiyon gözlenmiştir.

BMD modelleme, SSRI'ların fosfolipitleri daha potent olarak indüklediğini doğrulamıştır. Medyan BMD'ler karşılaştırıldığında SER'in nötral lipitlere kıyasla fosfolipitleri neredeyse 10 kat daha etkili şekilde indüklediğini görülmüştür. Ek olarak, SER ile fosfolipitler üzerindeki etki, bildirilen SSC aralığında (0.065–0.65  $\mu$ M) gözlenmiştir (Baumann 1996; De Vane et al. 2002). CIT, fosfolipit kanalında güçlü bir indüksiyon göstermesine rağmen, BMD modelleme bu etkinin nötral lipitlere benzer bir düzeyde olduğunu ve SSC aralığının (0.12–0.92  $\mu$ M) üzerinde olduğunu göstermiştir (De Vane et al. 2002).

Takiben, adipojenez sırasında lizozomlar üzerindeki etkiler lizozom spesifik boyama ile araştırılmıştır. Pozitif kontrol olarak 10  $\mu$ M AMIO kullanılırken, CIT ve SER sırasıyla 10–30  $\mu$ M ve 3,3–10  $\mu$ M'de test edilmiştir. Deney 2D protokole uygun

gerçekleştirilmiş, 14 günün sonunda adipositler boyanarak LysoTracker şiddeti floresans plaka okuyucuyla ölçülmüştür (Ex/Em 565/599 nm). SSRI'lar test edilen konsantrasyonlarda lizozomları indükleyici etki göstermiş, bu indüksiyonun pozitif kontrol AMIO düzeyinde olduğu görülmüştür.

Son olarak SSRI'ların olgun adipositlerde lipit profili üzerindeki etkileri araştırılmıştır. MKH'ler 14 gün boyunca pozitif kontrol ROSI (0.5  $\mu$ M) ile olgun adipositlere farklılaştırılmış, 14 günün sonunda test maddeleri uygulanarak (CIT: 30  $\mu$ M, SER: 10  $\mu$ M) deney 7 gün daha sürdürülmüştür. 21. günün sonunda adipositler boyanarak (lipit damlacıkları: Nile Red, hücre çekirdeği: Hoechst 33342), Nile Red şiddeti floresans plaka okuyucuyla farklı dalga boylarında ölçülmüştür. CIT ve SER'in olgun adipositlerde nötral lipit düzeyini etkilemediği, fakat fosfolipitleri artırdığı görülmüştür. Bu sonuç, ilaçların fosfolipitleri indükleyici etkisinin olgun adipositlerde de gerçekleştiğini göstermektedir.

### ***3T3-L1'lerle gerçekleştirilen in vitro adipojenez deneyi***

3T3-L1 hücreleri 10 % FBS ve 1 % Pen-Strep içerek DMEM'de (hücre ortamı) sürdürülmüş, *in vitro* adipojenez deneyleri pasaj 6-10 arasında gerçekleştirilmiştir. Yöntemin kısmi validasyonu referans bileşik *PPAR* $\gamma$  agonisti rosiglitazon (ROSI) ile gerçekleştirilmiş, 1  $\mu$ M ROSI deneylerde pozitif kontrol, 0.1 % DMSO ise taşıyıcı kontrol olarak kullanılmıştır. 3T3-L1 hücrelerinin farklılaşması 0.5 mM IBMX, 1  $\mu$ M Dex ve 5  $\mu$ g/mL insulin içeren hücre ortamı (farklılaşma mediumu) ile indüklenmiş, deney farklılaşma mediumu her 2 günde bir tazelenerek 8 gün boyunca sürdürülmüştür. 8. günün sonunda olgun adipositler Oil Red O ile boyanarak lipit birikimi plaka okuyucuyla ve mikroskopik incelemeyle değerlendirilmiştir.

Adipojenez deneylerini takiben ilaçların adipojenik protein ekspresyonu üzerindeki etkileri western blot yöntemiyle araştırılmıştır. Adipojenezin regülatörleri *PPAR* $\gamma$ , *C/EBP* $\alpha$ , pro-adipojenik transkripsiyon faktörü *SREBP1* ile *PPAR* yolağında bulunan *LPL* ve *FAS* protein miktarları belirlenmiştir. ROSI (1  $\mu$ M) pozitif kontrol olarak kullanılmış, CIT ve SER sırasıyla 30 ve 10  $\mu$ M konsantrasyonlarında test edilmiştir.

Beklendiği üzere ROSI adipojenik protein ekspresyonunu anlamlı şekilde artırmış, en güçlü etki adipojenezin ana regülatörü PPAR $\gamma$  ve PPAR $\gamma$  hedefi LPL üzerinde gözlenmiştir.

SSRI'lar test edilen konsantrasyonlarda lipit birikimini indükleyici etki göstermemiş, fakat test edilen konsantrasyonlarda adipojenik protein ekspresyonunu etkiledikleri gözlenmiştir. CIT, PPAR $\gamma$  ve C/EBP $\alpha$  ekspresyonunu artırırken, SER'in genel olarak protein ekspresyonunu inhibe ettiği görülmüştür.

Oil Red O boyamayla SSRI'larla lipit birikimi üzerinde anlamlı etkiler gözlenmemiş olsa da adipojenik protein ekspresyonundaki değişim CIT ve SER'in adipojenez üzerinde etkili olduğuna işaret etmektedir.

### ***Adipojenik etki mekanizmasının araştırılması***

Tez çalışması kapsamında ikinci hedefimiz SSRI'ların obezojenik etkilerinin altında yatan mekanizmaların *in vitro* yöntemlerle aydınlatılmasıdır. Obezite gelişiminde rol alan birçok hormonal yolak ve mekanizma söz konusudur. Çalışma kapsamında, adiposit farklılaşmasında rol alan çeşitli nükleer reseptörler aracılı mekanizmalara odaklanılarak, reseptör aracılı etkiler *in vitro* yöntemlerle araştırılmıştır.

İlk olarak, *in vitro* reseptör bağlanma ve transaktivasyon yöntemiyle ilaçların nükleer reseptörler üzerindeki agonist/antagonist aktivitesi test edilmiştir. Adipojenezin ana regülatörlerinden olan PPAR $\gamma$  ve PPAR $\alpha$  aracılıklı etkiler, reseptör eksprese eden haberci hücre hatları (HG5LN-PPAR $\gamma$  ve HG5LN-PPAR $\alpha$ ) kullanılarak araştırılmıştır. Özetle, hücreler 96 kuyucuklu plakalara ekilerek 24 saat inkübe edilmiştir. 24 saat sonunda hücre ortamı, test maddelerini içeren, hormon içermeyen hücre ortamı ile değiştirilerek 24 saat inkübe edilmiştir. İnkübasyon süresi sonunda hücrelerde reseptör aktivasyonunu takiben artan lusiferaz aktivitesi, luminesans şiddeti ölçülerek belirlenmiştir. İlaçların PPAR $\gamma$  veya PPAR $\alpha$  üzerine agonistik/antagonistik etkileri gözlenmemiştir.

Adiposit farklılaşması üzerinde etkili olduğu gösterilen ER, AR ve AhR (dioksin reseptörü -DR- olarak da adlandırılır) üzerindeki agonist/antagonist etkiler aynı yöntemle araştırılmıştır. Özetle, hücreler (ER: VM7Luc4E2, AR: T47D-ARE, DR: H1G1.1c3) 96 kuyucuklu plakalara ekilerek 48 saat inkübe edilmiştir. 48 saat sonunda

hücre ortamı, test maddelerini içeren, hormon içermeyen hücre ortamı ile değiştirilerek 24 saat inkübe edilmiştir. İnkübasyon süresi sonunda hücrelerde reseptör aktivasyonunu sonucu artan lusiferaz/fluoresans aktivitesi, luminesans/fluoresans şiddeti ölçülerek belirlenmiştir. İlaçlara ait ER, AR veya DR agonist/antagonist etki gözlenmemiştir.

İlaçların östrojenik etkileri ek olarak östrojen reseptörü pozitif (ER+) MCF7 meme kanser hücrelerinin hormon-bağımlı proliferasyonu ve östrojen sentezinde rol alan aromataz enzimi üzerindeki etkileri test edilerek belirlenmiştir.

Hormon-bağımlı proliferasyon deneyinde MCF7 hücreleri 96 kuyucuklu plakalara ekilerek 24 saat inkübe edilmiştir. 24 saat sonunda hücre ortamı test maddelerini içeren, östradiol varlığında veya östradiolsüz, hormon içermeyen mediumla değiştirilmiş, hücreler 144 saat inkübe edilmiştir. İnkübasyon süresinin sonunda hücre proliferasyonu sülforodamin B boyama yöntemiyle belirlenmiştir. İlaçlarla östradiolsüz ortamda MCF7 proliferasyonunda artış (ER agonistik etki), veya östradiol varlığında MCF7 proliferasyonunda azalma (ER antagonist etki) gözlenmemiştir. Bulgular, ER bağlanma ve transaktivasyonu deneyinin sonuçlarını doğrular niteliktedir.

İlaçların steroidojenez yolağının son basamağında görev alan ve testosteronun östrojene dönüşümünü katalizleyen aromataz enzimi üzerine etkileri, rekombinant aromataz enzimi ve enzimin floresans substratı 7-metoksi-4 triflorometil kumarinin (MFC) kullanılarak araştırılmıştır. İlaçların aromataz enzimi üzerine etkileri floresans şiddeti ölçülerek belirlenmiş, CIT ve SER ile test edilen konsantrasyonlarda aromataz enzimi üzerinde anlamlı bir etki gözlenmemiştir.

Özetle, CIT ve SER'in obezojenik etkileri *in vitro* adipojeniz yöntemiyle, 3T3-L1 fare preadiposit hücreleri kullanılarak araştırılmıştır. Oil Red O boyamayla SSRI'larla 3T3-L1 farklılaşmasında anlamlı bir artış gözlenmemiş olsa da western blot analizi SSRI'ların test edilen konsantrasyonlarda (CIT: 30  $\mu$ M, SER: 10  $\mu$ M) adipojenik protein ekspresyonunu etkilediği göstermiştir. Adipojenik protein ekspresyonundaki değişim CIT ve SER'in 3T3-L1'lerde adipojeniz üzerinde etkili olduğuna işaret etmektedir.

Bulguların yorumlanmasında 3T3-L1 hücre hattının zayıflıklarının göz önünde bulundurulması önem taşımaktadır. Adipojenik etkinin belirlenmesinde en yaygın

kullanılan hücre hattı olmasına rağmen, 3T3-L1'lerden elde edilen sonuçların hücrelerin elde edildiği kaynaklara göre farklılık gösterdiği literatürde bildirilmektedir (Kassotis et al. 2021). Bunun yanı sıra, 3T3-L1 hücre hattının adiposit soyuna bağlı olması nedeniyle bu yöntem sadece adiposit farklılaşmasının değerlendirilmesinde kullanılabilir. Öte yandan, insan MKH'leri multipotent kökenlerinden ötürü çeşitli hücre tiplerine farklılaşma özelliğine sahiptir. Bu özellikleri, adiposit farklılaşmasının yanı sıra hücrelerin adiposit soyuna bağlılığının değerlendirilmesine olanak vermektedir (Kassotis et al. 2022; Legler et al. 2020).

Bu nedenle, SSRI'ların obezogenik etkilerini daha kapsamlı bir şekilde değerlendirmek adına, CIT ve SER'in MKH'lerde adiposit farklılaşması üzerine etkileri 2D ve 3D modeller kullanılarak araştırılmıştır. 3D sistemlerin, yaygın olarak kullanılan 2D monolayer kültürlerle kıyasla iyileştirilmiş adiposit farklılaşması ve gen ekspresyonu ile *in vivo* koşulları daha iyi temsil ettiği literatürde bildirilmektedir (Klingelhutz et al. 2018; Muller et al. 2019; Shen et al. 2021). Bu nedenle, öncelikle SSRI'ların adipojenez üzerindeki etkilerini değerlendirmek için *in vivo* adipoz dokuyu daha iyi temsil eden bir 3D model geliştirilmiştir. Bu 3D modelde, literatürdeki çalışmalardan farklı olarak, adiposit progenitör hücreler yerine multipoten MKH'lerin kullanılması diğer çalışmalara göre üstünlük sağlamaktadır.

3D yöntemin optimizasyonu sırasında *PPAR $\gamma$*  agonisti ROSI referans bileşik olarak kullanılmıştır. Kontrol (DMSO) ve ROSI grubuna ait sferoidlerin morfolojileri H&E boyama ile histolojik analizlerle ve konfokal mikroskopisi ile belirlenmiştir. 3D sistemlerdeki en önemli endişe kaynaklarından biri, sferoidin merkezine doğru azalan oksijen difüzyonu nedeniyle hipoksik bir çekirdek oluşmasıdır (Trayhurn, Wang, and Wood 2008). H&E boyama ve konfokal mikroskopisi, sferoidlerde herhangi bir nekroz belirtisi olmaksızın lipid damlacıklarının homojen bir şekilde dağıldığını göstermiştir. Bu, literatürdeki sferoid modellerine kıyasla modelimizdeki sınırlı hücre sayısının nutrientlerin ve oksijenin hızlı difüzyonuna imkan sağlamasıyla açıklanabilir (Schmitz et al. 2021). Ayrıca, ROSI uygulaması lipid birikimi ve sferoid boyutunu artırmış, bu etkiyi hücre proliferasyonunda belirgin bir artışa neden olmaksızın göstermiştir. Fenotip olarak sferoidlerin 2D adipositlerden farklılık gösterdiği, özellikle ROSI grubu sferoidlerde *in vivo* adipoz dokuya benzerlik gösteren daha az sayıda ve daha büyük lipid damlacıklarının olduğu görülmüştür.

RNA-seq analizi, ROSI uygulaması ile her iki modelde de metabolik yolların benzer şekilde etkilendiğini göstermiştir. 3D modelde insulin ve adipokin sinyalizasyonu ile hücre yapılanmasıyla ilgili yolların daha güçlü etkilendiği görülmüştür. Bunun yanı sıra, modeller kıyaslandığında, 3D modelde biyolojik yolların genel olarak 2D modele göre downregüle olduğu görülmüştür. *PPAR $\gamma$*  ekspresyonunu ve *CEBP $\beta$*  aktivasyonunu etkileyerek adiposit farklılaşmasını inhibe ettiği bilinen TGF-beta sinyal yolağı da dahil olmak üzere birçok yolak, 3D modelde daha güçlü bir şekilde downregüle edilmiştir (Chen et al. 2016). TGF-beta sinyalizasyonundaki güçlü inhibisyonun, 3D modelde adipojenizin artan indüksiyonuna yol açabileceği düşünülmüştür. RNA-seq bulguları, literatürde bildirilen iyileştirilmiş adiposit farklılaşması ve gen ekspresyonunu destekleyerek, 3D modelin adipojenik etkinin test edilmesinde daha uygun bir ortam sağladığına işaret etmektedir (Shen et al. 2021).

3D modelin performansını değerlendirmek için adipojenik potansiyellerine göre seçilen bir grup obezojen, paralel 2D ve 3D deneylerde test edilmiştir. Obezojenlerle her iki modelde adiposit farklılaşmasında benzer bir indüksiyon gözlemlenmiştir. BMD modelleme, adipojenizin test edilmesinde 3D modelin 2D modele benzer etkinliğe sahip olduğunu doğrulamış, bu ise yeni 3D modelin kimyasalların adipojeniz üzerindeki etkilerini değerlendirmede uygulanabilir bir yöntem olduğunu kanıtlamıştır. Ancak QPCR analizi, obezojenler ile görülen indüksiyonun altında yatan gen ekspresyonu profilinin modeller arasında önemli farklılıklar gösterdiğini ortaya konmuştur. Gen ekspresyonundaki bu farklılıklar, 3D modelin *PPAR $\gamma$*  aktivasyonu dışındaki mekanizmalar aracılığıyla etki gösteren obezojenlere daha duyarlı olabileceğine ve bu durumun 2D modelde belirgin olmaksızın daha kompleks 3D sistemlerde ortaya çıkabileceğine işaret etmektedir. Özetle, 3D model, *in vivo* adipoz dokuya daha benzer bir fenotip ve adipojenik uyarılara karşı artan hassasiyet göstermiştir. Bu modeli karakterize etmek için insan adipoz dokusu ile doğrudan bir karşılaştırma bir sonraki adım olmasına rağmen, yapılan analizler 3D modelin SSRI'ların adipojeniz üzerindeki etkilerinin test edilmesinde uygulanabilir olduğunu göstermektedir.

SSRI'lar 3D modelde ilk olarak lipit birikimini indükleyerek adipojenik etki göstermiştir. Bu indüksiyon doza bağımlı şekilde ve referans bileşik ROSI ile görülen seviyede gözlenmiştir. BMD modelleme ile, bu etkinin literatürde belirtilen SSC

değerleri aralığında olduğu ortaya konmuştur (CIT: 0.12–0.92  $\mu$ M, SER: 0.065–0.65  $\mu$ M) (Baumann 1996; De Vane et al. 2002).

Tez çalışması kapsamında bir diğer hedefimiz SSRI'ların obezogenik etkilerinin altında yatan mekanizmaların aydınlatılmasıdır. CIT ve SER'in, adiposit farklılaşmasında rol alan çeşitli nükleer reseptörler üzerindeki agonist/antagonist aktivitesi *in vitro* reseptör bağlanma ve transaktivasyon yöntemiyle belirlenmiştir. İlaçların adipojenizin ana regülatörü olan PPAR $\gamma$  veya PPAR $\alpha$ 'yı aktive edici etkisi gözlenmemiştir. Hayvan çalışmalarında, östrojenik obezogenlere prenatal maruziyetin, ER aktivasyonu aracılığıyla, yavrularda obezite gelişimine neden olduğu gösterilmiştir (Darbre, 2017; Heindel et al., 2022; Newbold et al., 2007). Ek olarak, dioksin benzeri aktiviteye sahip bazı obezogenlerin, PPAR $\gamma$  ekspresyonunu dolaylı olarak değiştirerek adipojenezi indüklediği gösterilmiştir (Casals-Casas and Desvergne, 2011). Bu bilgilerden yola çıkarak, ilaçların ER, AR ve DR üzerindeki agonist/antagonist aktivitesi *in vitro* reseptör bağlanma ve transaktivasyon deneyleriyle araştırılmıştır. Ek olarak ER eksprese eden MCF7 hücrelerinin proliferasyonu ve steroidojenez yolağının son basamağında görev alan ve testosteronun östrojene dönüşümünü katalizleyen aromataz enzimi üzerine etkileri test edilerek ilaçların östrojenik etkisi değerlendirilmiştir. Transaktivasyon deneylerinde ilaçların ER, AR veya DR üzerinde agonist/antagonist etkisi gözlenmemiştir. SSRI'lar MCF7 proliferasyonu indükleyici (ER agonistik etki), veya östradiol varlığında MCF7 proliferasyonunu inhibe edici (ER antagonist etki) etki göstermemiş, ER transaktivasyon deneyinin sonuçları bu şekilde desteklenmiştir.

İlaçların rekombinant aromataz enzimi üzerinde anlamlı bir etkisi gözlenmemiştir. SSRI'ların aromataz enzimini inhibe edici etkileri bir çalışmada iki farklı mikrozom temelli *in vitro* yöntem ile gösterilmiştir (Jacobsen et al. 2015). Görülen inhibisyonun derecesi iki yöntem arasında değişiklik gösterse de, sonuçlar her iki SSRI'nın da enzim üzerinde inhibe edici etkiye sahip olduğunu göstermektedir. Ancak, çalışmada test edilen konsantrasyonların geniş bir aralığı kapsadığı, logaritmik aralıklı sekiz konsantrasyon seçildiği görülmektedir. Dolayısıyla, bildirilen etkiler bu tez çalışmasında test edilen ve fizyolojik konsantrasyon aralığında yer alan konsantrasyonlardan daha yüksek konsantrasyonlarda meydana gelmiştir. Deneylerimizde SSRI'larla aromataz inhibisyonunun görülmemesi, test edilen konsantrasyonlar arasındaki bu farklılıkla açıklanabilir. Bununla beraber, tüm bulgular

endokrin modülasyonun dışında bir mekanizmanın adipojenik etkileri tetiklediğine işaret etmektedir.

Adipojenik etkinin altında yatan mekanizmaların aydınlatılması için MKH'lerde RNA dizilimi analiz edilmiştir. RNA-seq analizinde SSRI'ların referans bileşik ROSI'ye kıyasla neredeyse tamamen farklı bir gen ekspresyonu profiline yol açtığı gözlenmiştir. Beklenmedik şekilde, adipojenik genler ve adiposit farklılaşmasında rol alan önemli yolların (PPAR sinyalizasyonu, adipojenez ve lipid metabolizması gibi) SSRI uygulaması sonucu downregüle olduğu görülmüştür. CIT ve SER'in adipojenezin regülatörleri (*PPAR $\gamma$* , *CEBP $\alpha$* ) ve PPAR sinyal yolağında bulunan hedef genlerin (*FABP4*, *ADIPOQ*) ekspresyonunu inhibe edici etkisi QPCR yöntemiyle de gösterilerek RNA-seq bulguları doğrulanmıştır.

Buna karşın, beklenmedik şekilde fosfolipit sentezi ve lizozomlarla ilişkili yolların SSRI uygulaması sonucu upregüle olduğu ve bu etkinin doza bağımlı olduğu gözlenmiştir. RNA-seq analizi ışığında, ilaçların adipojenez sırasında farklı lipid profilleri üzerindeki ve lizozomları indükleyici etkileri 2D MKH'lerde ek analizlerle araştırılmıştır. Deneyler sonucunda CIT ve SER'in 2D modelde fosfolipitleri ve lizozomları indükleyici etkisi gösterilerek RNA-seq bulguları doğrulanmıştır. BMD modelleme SSRI'ların fosfolipitleri doza bağımlı ve daha potent şekilde indüklediğini göstermiştir. Medyan BMD'ler karşılaştırıldığında SER'in nötral lipitlere kıyasla fosfolipitleri neredeyse 10 kat daha etkili şekilde indüklediği görülmüştür. Ek olarak, SER'in fosfolipitleri indükleyici etkisinin literatürde bildirilen SSC değerleri aralığında (0.065–0.65  $\mu$ M) olduğu bulunmuştur (De Vane et al. 2002).

SSRI tedavisi alan hastaların çoğunlukla yetişkinler olduğu göz önünde bulundurularak, ilaçların olgun adipositlerde lipid profili üzerindeki etkileri araştırılmıştır. CIT ve SER'in olgun adipositlerde nötral lipid düzeyini etkilemediği, fakat fosfolipitleri indüklediği görülmüştür. İlaçların fosfolipitleri indükleyici etkisinin olgun adipositlerde de gözlenmesi bu ilaçları kullanan hastalarda benzer etkilerin görülebileceğine işaret etmektedir.

Sonuç olarak bütün bulgular göz önüne alındığında, verilerimiz CIT ve SER'in katyonik amfifilik ilaçlar (KAİ'ler) olarak sınıflandırılan bir grup ilaçla benzer olumsuz etkilere yol açtığı şeklinde yorumlanmıştır. KAİ'ler ortak bir kimyasal yapıya



sahip olup zayıf bazik özelliklerinden ötürü lizozomlarda akümüle olankimyasallar olarak tanımlanabilir. Bu kimyasallar, yapılarında hidrofobik bir fonksiyonel gruba bağlı hidrofilik, protonlanabilir bir amin grubu taşımaktadır. Hidrofobik grubunun neden olduğu lipofiliteleri sayesinde hücre membranlarından kolaylıkla difüze olan KAI'ler lizozomlar gibi asidik bir organel içine girdiklerinde amin grubunun protonlanmasıyla pozitif olarak yüklenmektedir (Kazmi et al., 2013). Pozitif yüklü (katyonik) form membranı aşamadığı için lizozomlar içerisinde birikmekte, fosfolipit membranlara bağlanarak lizozomal enzimlerin inhibisyonuna yol açmaktadır. KAI'lerin lizozomlarda akümüasyonu, lizozomal fosfolipaz A2 (LPLA2) enziminin kompetitif inhibisyonuna yol açar, bunun sonucunda ise fosfolipit yıkımı sekteye uğrayarak lizozom içinde fosfolipit birikimi görülür (Hinkovska-Galcheva et al., 2021). KAI'lerin bu olumsuz etkisi literatürde hepatosit ve akciğer hücrelerinde gösterilmiş, ilaçların bu etkisi ilaçlarla indüklenen fosfolipidozis (DIP) olarak tanımlanmıştır (Hinkovska-Galcheva et al., 2021; Reasor et al., 2006).

CIT ve SER'in fizikokimyasal özellikleri hakkında literatürdeki bilgiler göz önüne alındığında (Reasor et al., 2006), çalışmamızın sonuçları ilaçların farklılaşan ve olgun adipositlerde lizozomal akümüasyonuna bağlı fosfolipidozise yol açtıklarına işaret etmektedir. RNA-seq sonuçlarında SSRI'larla LPLA2 inhibisyonuna dair bir veri bulunmamakla birlikte bu, LPLA2 inhibisyonunun moleküler düzeyde gerçekleşmesi ve gen ekspresyonundaki değişikliklerden bağımsız olmasıyla açıklanabilir. KAI'lerin 3T3-L1 farklılaşması üzerine etkilerinin araştırıldığı bir *in vitro* çalışmada ilaçların lizozomal akümüasyonu ile adipojenez inhibisyonu arasında pozitif bir ilişki bildirilmiştir (Kagebeck et al. 2018). Çalışmanın 3T3-L1 farklılaşmasının KAI'lerin lizozomlarda artan birikimi sonucu inhibe edildiğine işaret eden bulguları, çalışmamızda SSRI'ların 3T3-L1 ve MKH'ler üzerindeki etkilerini doğrular niteliktedir. 3T3-L1 hücreleriyle gerçekleştirilen bir başka *in vitro* çalışmada, KAI'lerin olgun adipositlerde fosfolipit membranlara bağlanma sonucu lizozomlarda akümüle olduğu gösterilmiş (Sanchez Garcia et al. 2018), çalışmamızda MKH'lerle yapılan olgun adiposit deneylerinin sonuçlarını doğrulamıştır. Tüm bulgular, CIT ve SER'in farklılaşan ve olgun adipositlerde fosfolipit membranlara bağlanarak lizozomlarda akümüasyonuna ve buna bağlı fosfolipit birikimini ve lizozomları indüklediğine işaret etmektedir.

Literatürde lizozomal disfonksiyon temelinde geliştirilen advers sonuç yolağı (adverse outcome pathway; AOP) yaklaşımları bulunmaktadır. Bunlar genellikle karaciğer toksisitesi üzerine geliştirilmiş olup, AOP144 ve AOP130 bunlara örnek olarak verilebilir (AOP144, lizozom disfonksiyonu; AOP130, fosfolipaz inhibisyonu) (Kuburic, Gerloff, and Landesmann 2023; Oh, Yoon, and Jegal 2023). Bu AOP'lerde bulunan çoğu anahtar olay (key event) çalışmamızın bulguları arasında mevcuttur. Lizozomal disfonksiyonun yanı sıra mitokondriyal disfonksiyon bu AOP'lerde ortak olarak bulunan bir diğer anahtar olaydır. Çalışmamızda RNA-seq verilerinde oksidatif fosforilasyon yolağının SSRI'larla etkilendiği görülmüştür. Bu, mitokondriyal fonksiyon üzerinde olası bir etkiye işaret etmektedir. KAİ'lerin hücrede lizozomların yanı sıra mitokondride akümüle olarak oksidatif fosforilasyonu inhibe ettiği literatürde bildirilmektedir (Fromenty, 2023). Kronik senaryoların test edildiği ileri deneylerle bu yolağın yol açabileceği inflamasyon ve hücre ölümü mekanizmalarının araştırılması, SSRI'ların advers etkilerinin kapsamının belirlenmesinde aydınlatıcı olacaktır.

Son olarak, SSRI'ların neden olduğu metabolik yolların downregülasyonunun altında yatan mekanizmanın aydınlatılması için, CIT ve SER'in *PPARγ* üzerindeki antagonistik etkileri araştırılmış, fakat ilaçların *PPARγ* antagonisti etkileri gözlenmemiştir. Adipojenezin ilk aşamasında *PPARγ* aktivasyonu membran fosfolipitlerinden araşidonik asit salınımıyla ilişkilidir. Sitozolik fosfolipaz A2 (cPLA2), araşidonik asit mobilizasyonu ve prostaglandin (PG) sentezinde önemli bir role sahiptir. Bu PG'lerden PG15d, *PPARγ* ligandı olarak aktivite göstererek adipojenik genlerin ekspresyonunu stimüle eder. Bu bağlamda cPLA2, adipojenezin erken aşamalarında proadipojenik bir faktör olarak rol almaktadır. Güncel çalışmalar adiposit farklılaşmasında fosfolipaz A2 yolağının önemini vurgulamaktadır (Peña et al. 2016). Literatürde bir *in vitro* çalışmada azitromisinin (KAİ grubu bir antibiyotik ajan) membran fosfolipitlerine bağlanmayı ve cPLA2 enziminin inhibisyonunu takiben PG sentezinde azalmaya neden olduğu bildirilmiştir (Banjanac et al. 2012). Literatürde bildirilen KAİ akümüasyonu ve adiposit farklılaşmasının inhibisyonu arasındaki pozitif korelasyon, KAİ'lerin *PPARγ* aktivasyonu ile sonuçlanan sinyalizasyon kaskadını inhibe etmesiyle açıklanabilir (Kagebeck et al. 2018). SSRI'ların MKH'lerde adipojenezi inhibe edici etkilerinin altında yatan mekanizma tam olarak aydınlatılamamakla birlikte, hipotezimiz CIT ve SER'in aynı

mekanizmayla etki gösterdiği, buna ek olarak, fosfolipit birikimi ve lizozom indüksiyonuna yol açarak adiposit fonksiyonunda bozulmaya neden olduklarıdır.

Sonuç olarak,

- 1) SSRI'ların obezogenik potansiyelinin araştırılmasında kullanılmak üzere MKH'lerde geliştirilen yeni 3D modelin, kimyasalların adipojenik etkilerinin taranması ve bu etkilerin olası mekanizmalarının aydınlatılmasında başarılı bir model olduğu gösterilmiştir. 3D model, adiposit progenitör hücre hatlarının kullanıldığı 2D modellere göre, adiposit farklılaşmasının iyileştirilmiş analizi ile insanlardaki durumun daha iyi yansıtılmasına olanak vermesi gibi önemli avantajlara sahiptir.
- 2) Bulgularımız, SSRI'ların adiposit farklılaşması ve olgun adipositlerde lipid metabolizması üzerindeki etkilerini kapsamlı olarak ortaya koymaktadır. Bu etkilerin, insanlarda bildirilen kararlı-durum plazma konsantrasyonları aralığında gözlenmesi, bulguların klinik önemini artırmaktadır. CIT ve SER'in neden olduğu fosfolipit ve lizozom homeostazının bozulması ile başlayan reaksiyon zincirinin, adiposit farklılaşmasının inhibisyonu ve adiposit fonksiyonun bozulmasıyla sonuçlandığı gösterilmiştir.
- 3) Çalışmamız, uzun süreli SSRI tedavisinin kilo artışına yol açtığını gösteren epidemiyolojik çalışmaların aksine, beklenmedik şekilde ilaçların adipojenezi baskılayıcı etkisini kapsamlı gen ekspresyonu analizleriyle ortaya koymuştur. *In vitro* sistemlerde gösterilen bu etkilerin *in vivo* sistemlerdeki karşılığına ilişkin kesin bir yargıya varmak güç olsa da, bulgularımız ilaçların metabolizma homeostazı üzerinde olası olumsuz etkilerine işaret etmektedir.

Tez çalışması kapsamında test edilen SSRI ilaçların adipojenez ve metabolizma üzerindeki advers etkilerinin ortaya konmuş olmasının obezite ve metabolik hastalıkların kompleks altyapısına sağladıkları katkının anlaşılması için önemli bir adım olduğu düşünülmektedir. Çalışmamız, SSRI gurubu antidepresanlar CIT ve SER'in yeterince araştırılmamış metabolik etkilerinin ve olası mekanizmalarının aydınlatılmasında önemli bir adım olup, ilaçların özellikle gebelik esnasında güvenli kullanımının sağlanması için bu etkilerinin ileri *in vitro* ve *in vivo* çalışmalarla araştırılmasının önemini vurgulamaktadır.

**Anahtar Kelimeler;** Sitalopram, sertralin, adipojenez, lizozom, fosfolipit

## **Abstract**

### **Investigation of endocrine disrupting and obesogenic effects of several pharmaceuticals that are exposed in the early stages of life**

Obesity has become a global health crisis, affecting both adults and children worldwide. While excessive calorie intake and genetic predisposition are recognized as major contributors to obesity, environmental factors, particularly chemicals known as 'obesogens', have gained attention for their potential role in this epidemic. Despite extensive research on environmental obesogens, the impact of pharmaceuticals, such as selective serotonin reuptake inhibitors (SSRIs), on obesity remains poorly understood. SSRIs like citalopram (CIT) and sertraline (SER) are widely prescribed, even during pregnancy; however, concerns have been raised about their effects on weight gain. This study investigated whether CIT and SER interfere with the process of adipocyte differentiation, using human mesenchymal stem cells (MSCs) and 3T3-L1 murine preadipocyte cell line, followed by an assessment of intracellular lipids by fluorescence staining and high-throughput/high-content analyses. A novel 3D model using MSCs was developed and characterized through transcriptomics analysis to improve the human relevance of the assay. Gene and protein expression analyses were performed to explore possible mechanisms, and receptor-mediated mechanisms were investigated using receptor binding and transactivation assays for various nuclear receptors, along with ER-dependent proliferation and cell-free aromatase inhibition assays. Alteration of lysosomal pathways revealed by transcriptional profiling was subsequently confirmed with functional read-outs in MSCs by an observed increase in lysosomes and phospholipids. Our findings suggest lysosomal dysfunction and disrupted lipid metabolism in mature adipocytes, leading to excessive lipid accumulation. Moreover, important adipogenic processes are inhibited, potentially leading to dysfunctional adipocytes, which might have implications for the maintenance of a healthy metabolic balance.

**Keywords;** Citalopram; sertraline; in vitro; adipogenesis; lysosome; phospholipid

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## Abbreviations

|                |                                      |
|----------------|--------------------------------------|
| ACN            | Acetonitrile                         |
| ADIPOQ         | Adiponectin                          |
| AMIO           | Amiodarone hydrochloride             |
| AR             | Androgen Receptor                    |
| AR-Luc         | AR Luciferase Assay                  |
| AhR            | Aryl hydrocarbon Receptor            |
| AOP            | Adverse outcome pathway              |
| APS            | Ammonium persulfate                  |
| ATCC           | American Type Culture Collection     |
| ATP            | Adenosine triphosphate               |
| $\beta$ -E2    | beta-Estradiol                       |
| $\beta$ -ME    | beta-Mercaptoethanol                 |
| BMD            | Benchmark Dose                       |
| BMI            | Body mass index                      |
| BMDL           | Lower bound of the BMD               |
| BMDU           | Upper bound of the BMD               |
| BPA            | Bisphenol A                          |
| BSA            | Bovine serum albumin                 |
| C/EBP $\alpha$ | CCAAT/enhancer binding protein alpha |
| cAMP           | Cyclic adenosine monophosphate       |



|                  |                                                 |
|------------------|-------------------------------------------------|
| cDNA             | Complementary DNA                               |
| CIT              | Citalopram hydrobromide                         |
| CO <sub>2</sub>  | Carbon dioxide                                  |
| Cq               | Cycle of quantification                         |
| CYP              | Cytochrom P450                                  |
| DCC              | Dextran-Coated Charcoal Stripped                |
| <i>p,p'</i> -DDE | <i>p,p'</i> - Dichlorodiphenyl-dichloroethylene |
| DDT              | Dichlorodiphenyltrichloroethane                 |
| DEGs             | Differentially expressed genes                  |
| DEHP             | Di-2-ethylhexyl phthalate                       |
| Dex              | Dexamethasone                                   |
| DMEM             | Dulbecco's modified Eagle's medium              |
| DMSO             | Dimethyl sulfoxide                              |
| DNA              | Deoxyribonucleic acid                           |
| DR               | Dioxin Receptor                                 |
| DTT              | Dithiothreitol                                  |
| EDC              | Endocrine disrupting chemical                   |
| EC50             | Effective Concentration 50                      |
| ECM              | Extracellular matrix                            |
| EDTA             | Ethylenediamine tetraacetic acid                |
| ER               | Estrogen receptor                               |

|        |                                                    |
|--------|----------------------------------------------------|
| ER-Luc | ER Luciferase Assay                                |
| ES     | Enrichment score                                   |
| EtOH   | Ethanol                                            |
| Ex/Em  | Excitation/Emission                                |
| FABP4  | Fatty acid binding protein 4                       |
| FBS    | Fetal bovine serum                                 |
| FCCP   | Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone |
| FDA    | U.S. Food and Drug Administration Agency           |
| FDR    | False discovery rate                               |
| FLUD   | Fludionil                                          |
| FLUT   | Flutamide                                          |
| GFP    | Green fluorescent protein                          |
| GO     | Gene ontology                                      |
| GSEA   | Gene set enrichment analysis                       |
| H&E    | Hematoxylin & Eosin                                |
| HEPES  | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HFC    | 7-hydroxy-4-trifluoromethyl coumarin               |
| IBMX   | 3-isobutyl-1-methylxanthine                        |
| IGF1R  | Insulin-Like Growth Factor 1 Receptor              |
| INSR   | Insulin Receptor                                   |
| KE     | Key event                                          |

|              |                                                             |
|--------------|-------------------------------------------------------------|
| KEGG         | Kyoto Encyclopedia of Genes and Genomes                     |
| KOH          | Potassium hydroxide                                         |
| Luc          | Luciferase                                                  |
| LPL          | Lipoprotein lipase                                          |
| MDCs         | Metabolism disrupting chemicals                             |
| MEHP         | Mono(2-ethylhexyl) phthalate                                |
| MEM $\alpha$ | Minimal essential medium alpha                              |
| MEM-NAA      | MEM Non-Essential Amino Acids Solution                      |
| MFC          | 7-methoxy-4-trifluoromethyl coumarin                        |
| MIE          | Molecular initiating effect                                 |
| MM           | Master mix                                                  |
| MSC          | Human bone-marrow derived mesenchymal stem cells            |
| NAFLD        | Non-alcoholic fatty liver disease                           |
| NaOH         | Sodium hydroxide                                            |
| Na-pyr       | Sodium pyruvate                                             |
| NES          | Normalized enrichment score                                 |
| OECD         | Organization for Economic Co-operation and Development      |
| ORA          | Over representation analysis                                |
| OXPHOS       | Oxidative phosphorylation                                   |
| P/S          | 10'000 Units/mL Penicillin + 10'000 $\mu$ g/mL Streptomycin |
| PBS          | Phosphate buffered saline                                   |

|                    |                                                  |
|--------------------|--------------------------------------------------|
| PCA                | Principal Component Analysis                     |
| PCB-128            | 2,3,4,2',3',4'-Hexachlorobiphenyl                |
| PFOA               | Perfluorooctanoic acid                           |
| PG                 | Prostaglandin                                    |
| PPAR $\alpha$      | Peroxisome proliferator-activated receptor alpha |
| PPAR $\alpha$ -Luc | PPAR $\alpha$ Luciferase Assay                   |
| PPAR $\gamma$      | PPAR gamma                                       |
| PPAR $\gamma$ -Luc | PPAR $\gamma$ Luciferase Assay                   |
| PVDF               | Polyvinylidene fluoride                          |
| QPCR               | Quantitative polymerase chain reaction           |
| RNA                | Ribonucleic acid                                 |
| RNA-seq            | RNA-sequencing                                   |
| ROSI               | Rosiglitazone                                    |
| RXR $\alpha$       | Retinoid X receptor alpha                        |
| SAT                | Subcutaneous adipose tissue                      |
| SD                 | Standard deviation                               |
| SDS                | Sodium dodecyl sulfate                           |
| SER                | Sertraline hydrochloride                         |
| SERT               | Serotonin transporter                            |
| SREBP1             | Sterol regulatory element-binding protein 1      |
| SSCs               | Steady state plasma concentrations               |

|       |                                     |
|-------|-------------------------------------|
| T2D   | Type 2 diabetes                     |
| TBBPA | Tetrabromobisphenol A               |
| TBT   | Tributyltin                         |
| TCA   | Trichloroacetic acid                |
| TCDD  | 2,3,7,8-Tetrachlorodibenzo-p-dioxin |
| TEMED | Tetramethylethylenediamine          |
| TESTO | Testosterone                        |
| TOCP  | Tri-ortho-cresyl phosphate          |
| TPP   | Triphenyl phosphate                 |
| US    | United States                       |
| VAT   | Visceral adipose tissue             |
| WHO   | World Health Organization           |

## 1. Introduction

Obesity, a condition characterized by excessive and unhealthy body weight, has evolved into a pandemic that is affecting people all around the world. In 2022, 43 % of adults aged 18 years and older were overweight and 16 % were obese, totaling up to 2.5 billion and 890 million people, respectively (WHO 2024). Furthermore, by 2022, 8 % of children and adolescents aged 5 to 19 were obese, totaling 160 million worldwide (WHO 2024), a number nearly double Turkey's entire population ((TÜİK) 2024).

Excessive calorie intake and genetic susceptibility are recognized as major factors contributing to obesity, yet these alone fail to fully explain this dramatic increase in obesity cases. This has led to a closer examination of the impact of environmental factors, specifically chemicals, on obesity development (Lustig et al. 2022). The "obesogen hypothesis," which emerged in the early 2000s, suggests chemicals can influence adipose tissue development and function through various mechanisms, to promote obesity (Grün et al. 2006). The obesogen field has grown substantially since then, with studies mostly focusing on identifying environmental chemicals acting as obesogens and providing a link between unintentional exposures and obesity (Heindel et al. 2022). However, despite a large number of pharmaceuticals being linked to significant weight gain, our understanding of their contribution to obesity development is limited and needs further research.

Selective serotonin reuptake inhibitors (SSRIs), are used for treating several mood disorders, and are among the most used medications worldwide (Serretti and Kato 2008). Citalopram (CIT) and sertraline (SER), two of the most prescribed SSRIs in pregnancy, have been linked to substantial weight gain in patients after long-term treatment (Arterburn et al. 2016; Blumenthal et al. 2014; Gafoor et al. 2018; Uguz et al. 2015). Additionally, there are reports on altered lipid profiles including increased serum triglyceride levels with CIT and total cholesterol levels with SER in female patients after 4 months of follow-up (Beyazyüz et al. 2013).

Although SSRIs are largely considered safe and prescribed to women during pregnancy, there is increasing concern related to their maternal and neonatal safety (Gill et al. 2020; Molenaar et al. 2020; Pariente et al. 2016). Given that CIT and SER are commonly prescribed to pregnant women, with a limited understanding of the

mechanisms underlying their metabolic effects, investigating these effects and the underlying mechanisms is timely.

In this context, our hypothesis in this thesis was that these SSRIs, CIT and SER, might have obesogenic effects, potentially through an endocrine mechanism. Furthermore, given their widespread use during pregnancy, they could potentially promote the worldwide increase in obesity.

The following literature review will first focus on the biology and the contributing factors behind obesity and metabolic diseases. Subsequently, it will introduce obesogens and their underlying mechanisms, highlighting specific chemicals that are classified as obesogens, continuing with various *in vitro* test methods used in the identification of obesogens. Lastly, the review will focus on Selective Serotonin Reuptake Inhibitors (SSRIs), their unintentional metabolic effects, and their potential role in obesity. The hypothesis of this thesis and the corresponding objectives will be presented based on all the reviewed information.

## 2. Literature Review

### 2.1. Obesity and Metabolic Diseases

Obesity is a chronic disease defined as “*an excessive accumulation of body fat that can impair health*” (WHO 2024). Over the last four decades, the incidence of obesity has nearly tripled, impacting adults and children all around the world. In 2022, obesity is observed in 890 million adults (18 years of age or older) and in 160 million children and adolescents (between 5-19 years of age) worldwide (WHO 2024). Now recognized as one of the most critical global health issues, obesity is not confined to developed countries; it is also a significant concern for developing nations (WHO 2024).

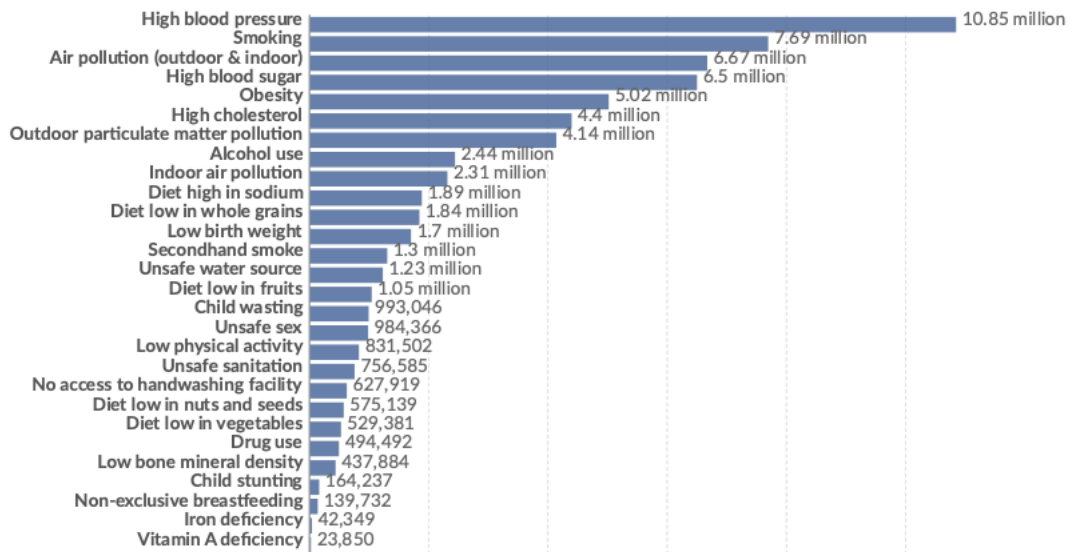
Besides being a serious health concern itself, obesity also increases the risk of metabolic syndrome, metabolic dysfunction-associated steatotic liver disease (MASLD), and various cancers (Lustig et al. 2022). Additionally, obesity leads to an elevated risk of premature mortality (Koroukian, Dong, and Berger 2019; Mathieu, Lemieux, and Després 2010). In 2019, obesity caused an estimated 5 million deaths from non-communicable diseases, including cardiovascular disease, stroke, diabetes, and various types of cancer, ranking fifth among risk factors related to premature death (Fig. 2.1). Interestingly, high blood pressure and high blood sugar are highly associated with obesity and part of metabolic syndrome ((NHLBI) 2022), which collectively account for another 17 million global deaths in 2019 (Fig. 2.1).



## Deaths by risk factor, World, 2019



The estimated annual number of deaths attributed to each risk factor<sup>1</sup>. Estimates come with wide uncertainties, especially for countries with poor vital registration<sup>2</sup>.



Data source: IHME, Global Burden of Disease (2019)

[OurWorldInData.org/causes-of-death](https://OurWorldInData.org/causes-of-death) | CC BY

**Figure 2.1** Estimated number of global deaths attributed to various risk factors. Obesity accounting for 8 % (approximately 5 million) of deaths globally (Ritchie and Roser 2017).

Obesity is typically assessed using the body mass index (BMI). BMI is calculated as the ratio of weight in kilograms to the square of height in meters ( $\text{kg/m}^2$ ). The World Health Organization (WHO) classifies a BMI greater than or equal to 25 as overweight, and a BMI exceeding 30 as obesity (WHO 2024). Despite being a widely used tool for evaluating obesity in adults of all ages and both sexes, BMI has its limitations. It primarily focuses on weight relative to height and lacks information about adiposity, a crucial aspect for distinguishing between 'healthy' and 'unhealthy' obesity, which is often associated with various metabolic disorders, *i.e.* metabolic syndrome (Lustig et al. 2022).

## ***Adipose Tissue***

Serving as the main energy store in the body, adipose tissue is a highly dynamic organ. It contains white, brown, and beige adipocytes, immune cells, endothelial cells, and fibroblasts (Qian, Tang, and Tang 2021).

White adipocytes have a high capacity for fat storage, limited vascularization, and few mitochondria. Found in fat depots throughout the body, white adipocytes are where excess energy is stored as triglycerides. These triglyceride stores are later broken down to free fatty acids and used during times of energy deprivation (Lustig et al. 2022). Besides acting as an energy depot, white adipose tissue also acts as an endocrine organ, secreting adipokines such as adiponectin (the insulin-sensitizing, anti-inflammatory, and anti-fibrotic hormone) and leptin, which play roles in regulating metabolism, appetite, and insulin sensitivity (Ghaben and Scherer 2019).

Conversely, brown and beige adipocytes are specialized for energy expenditure through thermogenesis. Brown adipocytes have little capacity for fat storage, are highly vascularized, and have high numbers of mitochondria. They express high levels of uncoupling protein 1 (UCP-1), which uncouples oxidative phosphorylation in the mitochondria to produce heat instead of ATP, as a defense against cold or as energy expenditure following food intake (Lustig et al. 2022). Beige adipocytes are primarily present in subcutaneous adipose tissue. In cases of prolonged cold exposure or exercise, white adipocytes can increase their mitochondria number and UCP-1 expression to become beige adipocytes, which appears to have a positive impact on metabolic health (Lustig et al. 2022). Overall, adipose tissue is crucial for energy homeostasis and regulating metabolism.

Adipose tissue development in humans occurs during *in utero* development and throughout childhood (Lustig et al. 2022). White adipocytes formed in early life tends to remain stable in adult life if body weight is maintained (Ghaben and Scherer 2019). However, excessive energy intake was shown to enhance both adipocyte number and size by increasing the maturation of fibroblast-like preadipocytes (Ghaben and Scherer 2019; Qian et al. 2021). This affects adipocyte function significantly, which in turn has a profound impact on metabolic health. Healthy adipose tissue is characterized by a high number of smaller white adipocytes, along with normal adiponectin secretion, normal response to insulin, functional mitochondria, proper vascularization, and

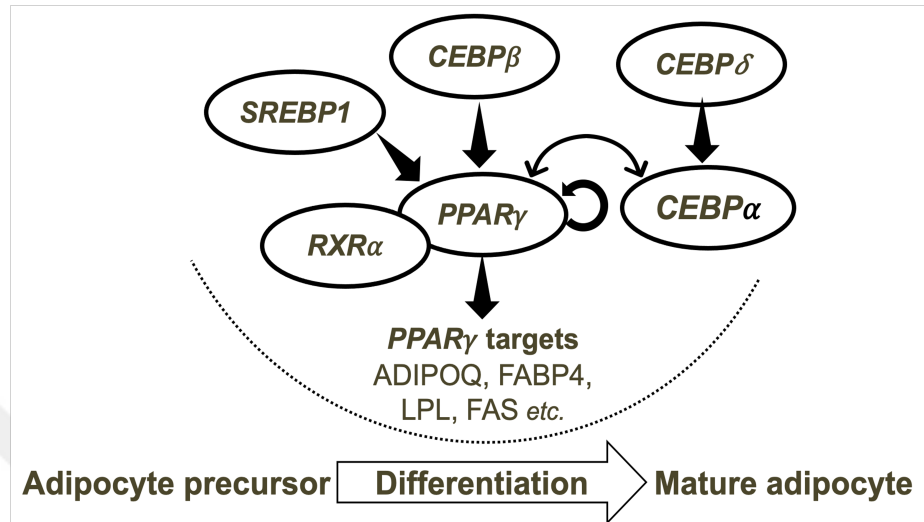
minimal macrophage infiltration. These allow for greater fat storage while adipocyte function is maintained, minimizing the risk of metabolic dysfunction (Lustig et al. 2022). On the other hand, in unhealthy obesity, adipocytes undergo hypertrophic expansion, and adiponectin secretion is decreased while leptin secretion is increased. Larger adipocytes undergo mechanical and hypoxic stress due to reaching the limit of oxygen diffusion and increased contact with neighboring cells. Hypoxia disrupts angiogenesis and increases pro-fibrotic gene expression which ultimately leads to tissue fibrosis. The heightened stress contributes to inflammation within the adipose tissue with elevated secretion of inflammatory cytokines (Halberg et al. 2009). Hypertrophy is also leads to increased insulin resistance and higher macrophage infiltration, exacerbating inflammation and metabolic disorders such as T2D and MASLD (Ghaben and Scherer 2019; Qian et al. 2021).

### ***Adipogenesis***

The precursor of adipocytes in the human body is the mesenchymal stem cell (MSC). The differentiation of MSCs into mature adipocytes is called adipogenesis. Various nuclear receptors acting as transcription factors regulate this intricate process, by directly modulating gene expression upon activation by a ligand (Lustig et al. 2022). Adipogenesis is initiated by the commitment of MSCs to the adipogenic lineage. Then, the key regulator of adipogenesis, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), is activated to stimulate terminal differentiation (Ghaben and Scherer 2019).

Adipogenesis is initiated with an increase in CAAT/enhancer-binding proteins beta and delta (CEBP $\beta$  and CEBP $\delta$ ), followed by an increase in PPAR $\gamma$  and CEBP alpha (CEBP $\alpha$ ). Activated PPAR $\gamma$  stimulates other transcription factors (Fig. 2.2), and forms a heterodimer with partner, retinoid X receptor alpha (RXR $\alpha$ ), leading to increased transcription of adipocyte genes and subsequent adipocyte differentiation (Janesick and Blumberg 2012; Lefterova and Lazar 2009; Sarjeant and Stephens 2012). Additional pro-adipogenic factors include sterol regulatory element-binding protein 1 (SREBP1), which induces PPAR $\gamma$  expression to promote adipogenesis. Some important PPAR $\gamma$  targets, commonly used as differentiation markers, include lipoprotein lipase (LPL), fatty acid synthase (FAS), fatty acid binding protein 4

(FABP4), leptin (LEP), and adiponectin (ADIPOQ) (Fig. 2.2) (Kamstra et al. 2014; Lefterova and Lazar 2009).

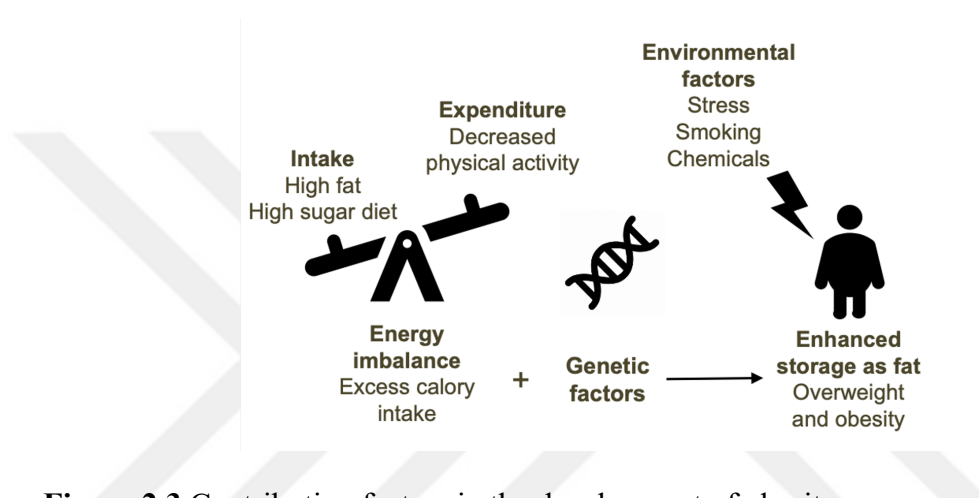


**Figure 2.2** Transcriptional regulation of adipogenesis. CEBP: CAAT/enhancer-binding protein, SREBP1: Sterol regulatory element-binding protein 1, PPAR $\gamma$ : Peroxisome proliferator-activated receptor gamma, RXR $\alpha$ : Retinoid X receptor alpha, ADIPOQ: Adiponectin, FABP4: Fatty acid binding protein 4, LPL: Lipoprotein lipase, FAS: Fatty acid synthase.

### ***Contributing Factors Behind Obesity***

Obesity is explained by the excessive consumption of calories, creating an imbalance between intake and expenditure. Excess calories are stored in the adipose tissue, leading to weight gain and obesity. Environmental and societal changes further exacerbate this imbalance by providing easier access to energy-dense foods and promoting sedentary lifestyles (WHO 2024). Genome-wide association studies have linked over 300 gene variants to obesity, which explains about 3–5 % of individual variation in the development of obesity (Schwartz et al. 2017). The pivotal roles energy imbalance and genetics play in promoting obesity cannot be overlooked. Nevertheless, they alone fail to fully explain the dramatic increase in obesity rates (Lustig et al. 2022;

Schwartz et al. 2017). This has shifted the focus onto environmental factors, especially chemicals as the cause of the current obesity pandemic (Figure 2.3). Substantial evidence from studies involving both animals and humans suggests environmental influences during fetal development can promote the onset of obesity and metabolic diseases during childhood and adulthood (Oken and Gillman 2003; Taylor and Poston 2007). Exposure to chemicals during development can predispose individuals to obesity later in life, potentially affecting multiple generations (Lustig et al. 2022).



**Figure 2.3** Contributing factors in the development of obesity.

The concept of chemicals' interference with the endocrine system has been known since the beginning of 1990s. The increase in endocrine-related diseases among humans and wildlife has led to the discovery of endocrine disrupting chemicals (EDCs). They are described as “*an exogenous substance or mixture that might interfere with the endocrine system, leading to adverse health effects in an intact organism, or its progeny, or (sub)populations*” (Bashshur, Mandil, & Shannon, 2002).

These chemicals exhibit various key characteristics, allowing them to influence the endocrine system through diverse mechanisms. These mechanisms include binding and activating hormone receptors (agonism), binding and blocking receptor activation (antagonism), altering receptor expression, interfering with hormonal signaling pathways, epigenetic modifications, and altering hormone kinetics (synthesis, transport, distribution, and metabolism). By binding and activating hormone receptors,

EDCs can replace endogenous hormones. However, for the compounds that interact with multiple receptors, their binding exhibits less specificity compared to their natural hormone counterparts. Due to this, their effects may lack the specificity observed with hormones binding to those same receptors (La Merrill et al. 2020). EDCs exhibit two other key characteristics setting them apart from most toxicants. EDCs are often effective at lower concentrations. Additionally, like endogenous hormones, they produce non-monotonic dose-response curves where effects at low concentrations differ from those at higher concentrations (Vandenberg et al. 2012). This response may be attributed to their impact on multiple endocrine pathways. At low concentrations, they might act as agonists or antagonists; however, at higher concentrations, negative feedback loops come into play, altering receptor sensitivity and/or expression, diminishing the agonist/antagonist response (Lagarde et al. 2015; Vandenberg et al. 2012).

As EDCs' effects were first discovered in wildlife and mainly on reproduction, studies to date mostly looked at the reproductive outcomes of environmental and industrial pollutants, through interference with steroid hormones (*e.g.*, estrogens, androgens) (A. Bergman et al. 2012; Gore et al. 2014). The research on EDCs has led to comprehensive screening strategies and regulations to address their impact on human and wildlife health. The European Union (EU) regulates EDCs based on data from various guidelines and programs released by the Organization for Economic Co-operation and Development (OECD), as part of the efforts to standardize and harmonize testing methods for the assessment of EDCs (Martyniuk et al. 2022). OECD uses a tiered approach to screen chemicals for their potential effects on the estrogen, androgen, and thyroid hormone systems, as well as steroidogenesis (referred to as the "EATS modalities"). Their approach includes predictive models (*e.g.*, *in silico* QSARs and ADME modeling) to detect substances that can affect the endocrine system, while a broad range of *in vitro* and *in vivo* assays help associate the chemical to any endocrine-related adverse effect ((OECD) 2018). The primary focus of the regulatory bodies has been on these EATS pathways. However, there's growing attention on the non-EATS pathways like metabolism and weight gain (Martyniuk et al. 2022). After the questioning of chemicals' (especially with endocrine-disrupting properties) impact on the global increase in obesity prevalence, the focus on reproduction has shifted towards metabolism disruption and obesity.

## **2.2.Obesogens and Mechanisms Underlying Obesogen Action**

The "obesogen hypothesis", which emerged in the early 2000s, proposes that some EDCs, later termed obesogens, can influence an individual's predisposition to obesity by disrupting the endocrine regulation of calorie intake (appetite, satiety), storage, expenditure, and adipose tissue development (Grün et al. 2006). Studies have supported this hypothesis, providing evidence on obesogens' interference with these hormonally driven processes, suggesting that exposure to obesogens can promote obesity (Casals-Casas and Desvergne 2011; De Cock and Van de Bor 2014; Retha R. Newbold et al. 2007).

Understanding the specific mechanisms through which these chemicals alter metabolic processes is essential to establish an irrefutable link between obesogens and obesity. Research in the obesogen field has focused on unveiling these mechanisms to identify the fundamentals of obesogen action. These include nuclear receptor-mediated effects on adipocyte differentiation, epigenetic modifications accounting for intergenerational and transgenerational effects, direct effects on certain organs regulating metabolism (*e.g.*, liver, adipose tissue, muscles, brain), and indirect mechanisms like inducing inflammation and mitochondrial/oxidative stress (Blumberg and Egusquiza 2020; Heindel et al. 2022).

### ***Receptor-mediated mechanisms***

Obesogens can act on several nuclear receptors involved in adipose lineage commitment and differentiation. The prominent targets are the ligand-activated transcription factors, peroxisome proliferator-activated receptors (*PPAR* $\gamma$ , *PPAR* $\alpha$ , and *PPAR* $\delta$ ), and retinoid X receptor alpha (*RXR* $\alpha$ , heterodimeric partner of *PPAR* $\gamma$ ). Among these, *PPAR* $\gamma$  and *RXR* $\alpha$  are vastly expressed in adipose tissue (Lustig et al. 2022). Liver transcription factors include liver X receptor (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR), and aryl hydrocarbon receptor (AhR). These can modulate hepatic lipid metabolism to direct lipids to adipose tissue for storage (Lustig et al. 2022). Lastly, systemic hormone receptor systems including insulin, estrogen, androgen, glucocorticoid, and thyroid

receptors (IR, ER, AR, GR, and TR) modulate energy storage and metabolism (Casals-Casas and Desvergne 2011; Lustig et al. 2022).

In this thesis, the focus was on the main target *PPAR* $\gamma$ , as well as the three receptors (AhR, ER, and AR) that were tested to evaluate possible receptor-mediated effects of the SSRIs.

### ***Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )***

*PPAR* $\gamma$  plays a pivotal role in adipocyte differentiation. Adipogenesis involves two phases: commitment and terminal differentiation. An increase in *PPAR* $\gamma$  expression marks the transition to the differentiation phase. Endogenous ligands, like fatty acids, eicosanoids, prostaglandins, activate *PPAR* $\gamma$ . Upon activation, *PPAR* $\gamma$  triggers a cascade of effects, such as stimulating transcription factors, and promoting adipogenic gene expression, leading to enhanced adipocyte differentiation (Ghaben and Scherer 2019). It is recognized today that most obesogens activate *PPAR* $\gamma$  in a relatively well-defined mechanism. There is strong evidence showing that *PPAR* $\gamma$  activation by several environmental chemicals promotes obesity, including phthalates, bisphenols, flame retardants, organofluorine compounds, and tributyltin (TBT) (Blumberg and Egusquiza 2020; Heindel et al. 2022). (Ahmed and Atlas 2016; Cano-Sancho, Smith, and La Merrill 2017; Feige et al. 2007; Grün et al. 2006; Kakutani et al. 2018; Watkins et al. 2015). Generally, *PPAR* $\gamma$  activation results in less inflammation, normal insulin/leptin secretion, therefore there is no accompanying metabolic disturbance initially (such as with thiazolidinediones) (Ghaben and Scherer 2019; Qian et al. 2021). Partial agonists often have a higher risk of causing a dysfunctional phenotype (Shoucri et al. 2018). Although the PPAR pathway is quite well-known, the knowledge on some of the other mechanisms is still very limited and can widely vary between chemicals (Blumberg and Egusquiza 2020; Heindel et al. 2022).

### ***Retinoid X receptor alpha (RXR $\alpha$ )***

*RXR* $\alpha$  forms a heterodimer with *PPAR* $\gamma$  and functions as a key regulator of adipogenesis (Lustig et al. 2022). Activation of *RXR* $\alpha$  promotes both preadipocyte commitment and adipogenic differentiation. Recent studies highlight *RXR* $\alpha$ 's crucial role in adipogenic lineage commitment (Shoucri et al. 2017), while adipocyte differentiation induced by *RXR* $\alpha$  was associated with a distinct adipocyte, compared



to the ones induced by *PPAR* $\gamma$ . RXR-induced adipocytes exhibited decreased glucose uptake and adiponectin expression, suggesting a dysfunctional adipocyte that may contribute to the risk of obesity and metabolic diseases (Shoucri et al. 2018).

### ***Aryl hydrocarbon receptor (AhR)***

The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, responds to environmental signals, particularly increased exposure to chemicals, and triggers adaptive responses such as detoxification and immune activation. Another common name for AhR is the dioxin receptor (DR), coming from its high affinity for dioxin-like substances. Unsurprisingly, the liver expresses high levels of AhR, given its role in detoxification (Beischlag et al. 2008). AhR signaling plays an important part in metabolic deregulation, and studies showed AhR inhibition can prevent and reverse obesity (Tanos et al. 2012). Furthermore, AhR has been shown to modulate adipogenesis, indirectly through the downregulation of *PPAR* $\gamma$  expression (Casals-Casas and Desvergne 2011; Darbre 2017), and the potent AhR ligand, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has been shown to inhibit adipogenesis *in vitro* in 3T3-L1 preadipocytes (Bastos Sales et al. 2013).

### ***Estrogen receptor (ER)***

Estrogens exert their effects through the nuclear receptors ER $\alpha$  and ER $\beta$ , and G-protein-coupled membrane receptors. Expression of both membrane and nuclear receptors has been shown in human adipose tissue (Hugo et al. 2008). Estrogen signaling seems to have diverse effects on adipocyte differentiation, partly depending on the receptors involved and the timing of exposure. In postmenopausal women, decreased estrogen levels correlate with enhanced adiposity and obesity, a condition reversible with hormone therapy (Casals-Casas and Desvergne 2011). Conversely, exposure to the synthetic estrogen diethylstilbestrol during development was shown as a risk factor for obesity (C. J. Hao et al. 2012; Hatch et al. 2014). Adipogenic effects of the estrogenic plasticizer bisphenol A (BPA) have been shown *in vitro* (Boucher, Boudreau, and Atlas 2014; Riu et al. 2011), and *in vivo*, including transgenerational effects such as higher adipose tissue mass in the offspring of mice (Susiarjo et al. 2015).

### ***Androgen receptor (AR)***

Androgens exert their effects through the nuclear AR. Reduced androgen levels and anti-androgen treatment have been linked to increased adiposity (Kassotis et al. 2017), leading to the general consideration of androgens as anti-obesogenic. However, there are notable knowledge gaps regarding AR signaling pathways and their implications for obesity, and further research is needed to clarify these effects (Venkatesh et al. 2022).

### ***Epigenetic mechanisms***

Epigenetics studies the environmental influences that can alter gene expression. Although epigenetic modifications occur without changes in the genome itself, they are heritable to the following generations, hence termed transgenerational effects. The most well-known epigenetic mechanisms include DNA methylation and modification of histone proteins surrounding the DNA, *e.g.*, histone methylation (Mohajer et al. 2021; Stel and Legler 2015). *In utero* development is a sensitive period for exposure to obesogens acting via epigenetic mechanisms, as these changes will likely impact future generations. This was shown in transgenerational studies, where exposure to obesogens tributyltin (TBT), bisphenol A (BPA), and dichlorodiphenyl-trichloroethane (DDT) during prenatal development led to a predisposition to obesity in non-exposed offspring (Chamorro-García et al. 2013; Skinner et al. 2013; Susiarjo et al. 2015).

### ***Other mechanisms***

Energy homeostasis and metabolism, thus body weight, are tightly controlled by the interactions between multiple organ systems, including adipose tissue, muscles, liver, and brain. Obesogens can act on a number of these organ systems to promote obesity (Shoucri et al. 2017).

Obesogens can promote lipid accumulation in the liver. Increased adiposity contributes to insulin resistance and inflammation, promoting MASLD, as well as more severe

forms of liver damage (Birkenfeld and Shulman 2014). Obesogens that worsen insulin resistance in the liver further exacerbate the risk of MASLD through this mechanism.

Obesogens can also promote obesity via indirect mechanisms. Most obesogens have been shown to disrupt the immune system and mitochondrial functions, leading to inflammation, and increased mitochondrial/oxidative stress (Bansal, Henao-Mejia, and Simmons 2018). Increased oxidative stress can activate inflammatory pathways and exacerbate the risk of metabolic abnormalities such as insulin resistance (Colak and Pap 2021). When obesogen exposure leads to chronic systemic inflammation or inflammation within organs regulating metabolism, there is a higher risk of metabolic disturbance (Ghaben and Scherer 2019; Heindel et al. 2022).

### **2.2.1. Chemicals Classified as Obesogens**

Currently, there are many chemicals and chemical classes suspected or classified as obesogens based on consistent evidence, including data from *in vitro*, mechanistic, and animal studies, along with human data linking exposure to adverse metabolic effects such as weight gain, higher BMI, and obesity in humans (Heindel et al. 2022).

The focus of this study was on a smaller group of obesogens (summarized here and shown in Fig. 2.5), with various mechanisms and substantial evidence on their impact on adipocyte differentiation.

#### ***Thiazolidinedione drugs***

Rosiglitazone (ROSI), troglitazone, and pioglitazone, antidiabetic agents belonging to the family of thiazolidinediones (TZDs), are originally developed for the treatment of T2D. Tailored to increase insulin sensitivity, they act as potent *PPAR* $\gamma$  agonists. Particularly ROSI has been shown to enhance adipocyte differentiation *in vitro* in 3T3-L1 cells and in MSCs via *PPAR* $\gamma$  activation (Grün et al. 2006; Kassotis et al. 2021; Legler et al. 2020). Due to its strong affinity for *PPAR* $\gamma$ , ROSI is commonly used as a reference chemical in *in vitro* adipogenesis assays for its strong adipogenic potential.

Next to their pharmacological action, TZDs are also associated with weight gain in patients (Dutta et al. 2023; Ko, Kim, and Lee 2017; Medici, McClave, and Miller 2015;

Wilding 2006). Although the mechanisms by which TZDs cause weight gain include fluid retention, decreased sodium excretion, and increased lipid storage (Basu et al. 2006; Guan et al. 2005; Nesto et al. 2004; Yang and Soodvilai 2008), *PPAR* $\gamma$  activation likely plays a part as well, by increasing the differentiation of precursors into adipocytes.

### ***Organotins***

The well-studied organotin TBT was commonly used in marine paints as an anti-fouling agent. However, due to its harmful effects on the environment and on human health, many countries have implemented regulations to restrict or ban its use in anti-fouling paints, which led to a global ban in 2008 (Lagadic et al. 2018). Now recognized as a prominent obesogen, TBT acts through nanomolar affinity binding to *PPAR* $\gamma$  and RXR, though now it is recognized as a partial agonist on *PPAR* $\gamma$  (Heindel et al. 2022). Its ability to enhance preadipocyte differentiation was shown both *in vitro* (Chamorro-García et al. 2013; Grün et al. 2006; Li, Ycaza, and Blumberg 2011) and *in vivo* (Chamorro-García et al. 2013; Chamorro-Garcia et al. 2017). Moreover, in transgenerational studies, prenatal TBT exposure was associated with increased adiposity in the F1, F2, and F3 descendants of F0 mice exposed during pregnancy (Chamorro-García et al. 2013; Chamorro-Garcia et al. 2017). Despite the lack of human data, substantial experimental data from both *in vitro* and *in vivo* assays strongly suggests the obesogenic potential of TBT (Heindel et al. 2022).

### ***Bisphenols***

Bisphenols are industrial plasticizers used in everyday items like water bottles and food containers (Veiga-Lopez et al. 2018). The well-known obesogen bisphenol A (BPA) was shown to enhance adipocyte differentiation possibly through *PPAR* $\gamma$  activation (Boucher et al. 2014; Riu et al. 2011). Transgenerational effects upon BPA exposure were shown *in vivo* in mice, via epigenetic mechanisms, including higher adipose tissue mass in male offspring, associated with epigenetic changes (Susiarjo et al. 2015). A recent *meta*-analysis identified 12 cross-sectional studies where a significant positive association was found between BPA exposure and obesity in adults (Ribeiro et al. 2020).

### ***Brominated flame retardants***

Brominated flame retardants, initially used for their flame-reducing properties, faced restrictions due to environmental and health concerns (Heindel et al. 2022). Tetrabromobisphenol A (TBBPA), a member of brominated flame retardants, was shown to activate *PPAR* $\gamma$  (Kakutani et al. 2018), and increase MSC differentiation into adipocytes (Riu et al. 2011).

Organophosphates, replacements of brominated flame retardants, have similar toxicity but relatively shorter half-lives (Heindel et al. 2022). The organophosphates triphenyl phosphate (TPP) and tri-ortho-cresyl phosphate (TOCP) were shown to increase adipocyte differentiation and modulate glucose uptake *in vitro* in 3T3-L1 cells (Cano-Sancho, Smith, et al. 2017; Liu et al. 2022). Prenatal and neonatal exposure to TPP in mice was shown to increase body weight, fat mass, and hepatic lipid accumulation in male offspring (Wang et al. 2018, 2019).

Despite the limited human data, both former brominated flame retardants, and their newer replacements, organophosphate compounds, are suspected obesogens through consistent experimental evidence (Heindel et al. 2022).

### ***Phthalates***

Di(2-ethylhexyl) phthalate (DEHP), an industrial plasticizer is the most well-studied member of phthalates. DEHP undergoes rapid conversion to mono(2-ethylhexyl) phthalate (MEHP) in the human body, which acts as a potent *PPAR* $\gamma$  agonist (Veiga-Lopez et al. 2018). MEHP was shown to induce preadipocyte differentiation *in vitro* in 3T3-L1 cells (Feige et al. 2007), and *in vivo*, leading to increased body weight and visceral fat mass in mice (C. Hao et al. 2012). Phthalate exposure was associated with obesity in a recent systematic review and *meta*-analysis (Ribeiro et al. 2020), and a prospective study found a significant link between high phthalate levels in women and weight gain (Song et al. 2005).

### ***Fludioxonil***

The fungicide fludioxonil (FLUD) has been identified as an *RXR* $\alpha$  agonist, and was shown to enhance adipogenesis *in vitro* in 3T3-L1 preadipocytes and in mouse bone marrow derived mesenchymal stem cells (Janesick et al. 2016). A more recent study

classified FLUD as a potential *PPAR* $\gamma$  ligand (Kim et al. 2021). While these findings support FLUD's potential as an obesogen, further investigation is needed to better understand its effects on adipogenesis and to establish a causal link between exposure and weight gain in humans.

### ***Organofluorine compounds***

Organofluorine compounds are per- and poly-fluoroalkyl substances (PFAS), a large group of chemicals widely used in manufacturing and in everyday consumer products for heat protection. Although their use was restricted in some countries following concerns about their persistence, bioaccumulation, and potential health effects, they are still found in the environment due to their widespread use and persistent nature (Schrenk et al. 2020).

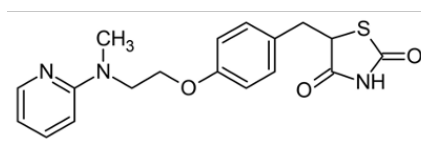
Previous *in vitro* research has shown PFAS to be activators of PPARs, including *PPAR* $\gamma$  and *PPAR* $\alpha$  (Behr et al. 2020; Evans et al. 2022; Houck et al. 2021; Nielsen et al. 2023). Perfluorooctanoic acid (PFOA), was found to enhance lipid accumulation and alter the expression of genes associated with adipocyte differentiation and lipid metabolism, including *PPAR* $\gamma$ , *in vitro* in 3T3-L1 cells (Ma et al. 2018; Watkins et al. 2015). Beyond *PPAR* $\alpha$ -dependent effects, studies revealed PFOA-induced outcomes such as increased body weight and hepatic lipid accumulation in both *PPAR* $\alpha$  knockout and wildtype mice (Attema et al. 2022; Das et al. 2017).

Epidemiological studies supported the obesogenic effects of PFOA. Cross-sectional studies reported a positive correlation between PFOA exposure and enhanced adiposity and a higher prevalence of overweight and T2D (He et al. 2018; Tian et al. 2019). However, findings from studies exploring the causal relationship between prenatal PFAS exposure and childhood obesity have been inconsistent. Some prospective cohort studies have linked higher prenatal PFOA exposure to greater weight and adiposity among children (Halldorsson et al. 2012; Lauritzen et al. 2018), while a systematic review of 13 cohorts examining prenatal PFAS exposure found a positive but non-significant association (Frangione et al. 2024).

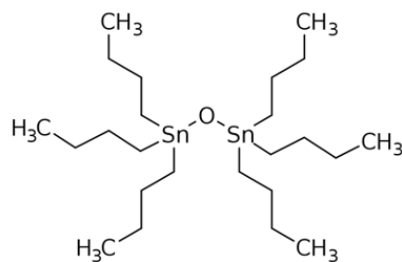
### ***Organochlorines***

Lastly, the infamous insecticide dichlorodiphenyltrichloroethane (DDT), known for its disastrous effects on reproduction, is also associated with adverse effects on

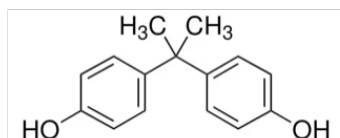
metabolism. Although DDT is restricted in most countries following concerns about its impact on the environment, wildlife, and human health, it is still used as an effective agent to control malaria (Å. Bergman et al. 2012). Given its persistent nature, DDT and its metabolite *p,p'*-dichlorodiphenyl-dichloroethylene (*p,p'*-DDE), which is even more lipophilic, can still be found in the environment (Darbre 2017). DDT and *p,p'*-DDE were shown to induce adipogenesis *in vitro* in 3T3-L1 cells, possibly through an estrogen-mediated mechanism (Kim et al. 2016; Mangum, Howell, and Chambers 2015). Prenatal DDT exposure was shown to increase body weight and abdominal adiposity in F3 descendants of exposed rats (Skinner et al. 2013). Given the widespread use of DDT, it was frequently screened in epidemiological studies, including studies of birth cohorts, and prospective studies. A systematic review of seven prospective studies found a positive correlation between prenatal *p,p'*-DDE exposure, and adiposity in children (Cano-Sancho, Salmon, and Merrill 2017). Conversely, a more recent review of two Belgian cohorts found no association between prenatal *p,p'*-DDE exposure and long-term child growth (Cai et al. 2023).



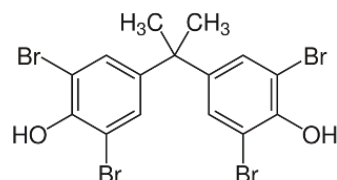
Rosiglitazone (ROSI)



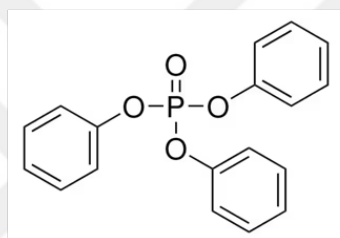
Tributyltin (TBT)



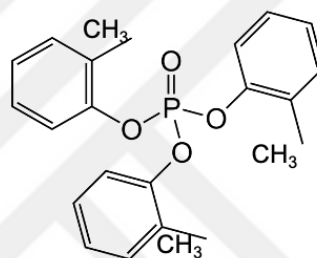
Bisphenol A (BPA)



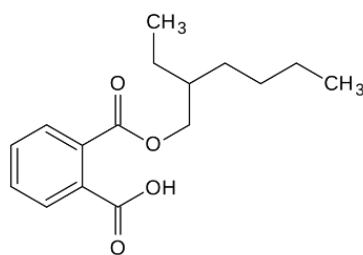
Tetrabromobisphenol A (TBBPA)



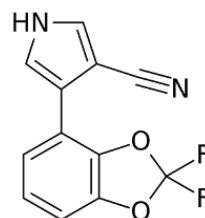
Triphenyl phosphate (TPP)



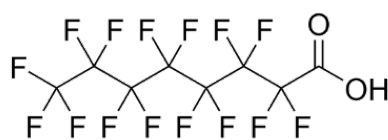
Tri-ortho-cresyl phosphate (TOCP)



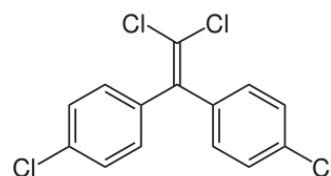
Mono(2-ethylhexyl) phthalate (MEHP)



Fludioxonil



Perfluorooctanoic acid (PFOA)



p,p'-Dichlorodiphenyl-dichloroethylene (ppDDE)

**Figure 2.4** The battery of obesogens tested in this study.



### **2.2.2. *In Vitro* Test Methods for Identifying Obesogens**

Given the complex nature of chemical exposure and the multifactorial setting of obesity, developing a robust set of testing methods for identifying obesogens is challenging. It is important to employ a weight-of-evidence approach when classifying a chemical as an obesogen. Inconsistencies between animal studies arise from differences in timing and routes of exposures, as well as measured endpoints. Similarly, challenges in accurately assessing human exposures along with additional confounding factors in epidemiological studies make it more difficult to establish causal relationships between chemical exposures and adverse health effects in humans (Heindel et al. 2022). Therefore, when evaluating these studies, careful interpretation of the data is needed. Furthermore, developing standardized and harmonized *in vitro* testing methods is crucial to move the obesogen field forward.

In 2019, the European research project GOLIATH started with the overall aim of generating an Adverse Outcome Pathway (AOP) for metabolism disruption, as well as a new Integrated Approach to Testing and Assessment (IATA) for chemicals that interfere with metabolic functions. This integrated approach aims to assess whether a chemical can be classified as a metabolism disrupting chemical (or an obesogen), based on a weight-of-evidence approach, by using data from literature supplemented with data from novel test methods targeting main nuclear receptors, cells, and tissues involved in metabolic disruption (Legler et al. 2020).

Summarized here are the *in vitro* test methods that were used in this study to evaluate the obesogenic effects of the SSRIs.

#### ***Receptor binding and transactivation assays***

Metabolism involves a complex interplay of molecular initiating events (MIEs) and key events (KEs) between multiple organs and tissues (Legler et al. 2020). Although there is no complete AOP on adipogenesis, one of the MIEs is identified as the activation of various nuclear receptors (Legler et al. 2020). Receptor binding and transactivation assays are widely used, high-throughput screening assays to predict nuclear receptor binding and activity in humans. These assays typically involve the use of stably transfected cell lines to evaluate whether a substance can activate or inhibit nuclear receptors. Cell lines used in these assays (also known as reporter cell

lines) are genetically modified to express a responsive luciferase reporter gene plasmid to induce luciferase activity upon activation of the receptor by a ligand. For instance, MIE screening assays for metabolism have been developed using reporter cell lines expressing the ligand-binding domain of human peroxisome proliferator-activated receptors PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$  (Seimandi et al. 2005).

Receptor binding and transactivation assays for the ER and AR are designed to detect chemicals that may act as agonists or antagonists. Guidelines for these assays have been released by the OECD as part of the efforts to standardize and harmonize testing methods for the assessment of EDCs (OECD 2021, 2023). Similar to ER binding and transactivation assay, ER-dependent proliferation assay is used to detect ER binding activity of chemicals. This assay involves the detection of downstream effects of ER activation (in this case, increased cell proliferation) by using hormone-responsive cells (*e.g.*, MCF7 (ER +) breast cancer cell line) (Soto et al. 1995).

### ***Enzyme activity assays***

Hormone synthesis is a common target for endocrine and metabolism disrupting chemicals. There are several cell-based and cell-free *in vitro* assays to detect chemicals' effects on the enzymatic production and/or breakdown of natural hormones. However, most of these assays are developed and standardized for the EATS pathways (see the end of chapter 2.1), and there is still a need for new test methods to screen chemicals that can alter non-EATS hormone signaling pathways (Martyniuk et al. 2022).

For instance, steroidogenesis and aromatase enzyme activity assays are widely used screening tools to detect substances with suspected estrogenic or androgenic activity. Aromatase enzyme (also known as estrogen synthase) is located in the last step of the steroidogenesis pathway and is essential for estrogen synthesis. The cell-free aromatase inhibition assay uses human recombinant aromatase enzyme and specific substrates to measure enzyme inhibition by chemicals (Jacobsen et al. 2015), which is used as an indication for antiestrogenic activity.

### ***In vitro adipogenesis assays***

As mentioned above, there are ongoing efforts to develop *in vitro* models targeting key tissues for metabolic disruption (*e.g.*, liver, pancreas, skeletal muscle, and adipose tissue). Currently, the focus for adipose tissue is on developing human-relevant models to test chemicals' effects on adipocyte differentiation, *i.e.*, adipogenesis assays (Legler et al. 2020). The number of available cell models to assess the effects on adipogenesis has been increasing over the past decade. The most established methods generally use adipogenic progenitor cells, such as the murine preadipocyte cell line 3T3-L1, or MSCs isolated from bone marrow or adipose tissue. The cells can be differentiated into mature adipocytes *in vitro* by exposing them to an adipogenic medium containing a mixture of differentiation factors. These factors include IBMX, dexamethasone, and insulin, which are responsible for the activation of key transcription factors involved in adipogenesis such as *PPAR $\gamma$*  and *C/EBP $\alpha$*  (Chen et al. 2016; Lustig et al. 2022). Generally, an increase in intracellular lipid accumulation is quantified and used as a measure for enhanced adipogenesis (Kassotis et al. 2022; Legler et al. 2020).

Although the 3T3-L1 cell line is robust and well-characterized, it provides limited information, as the cells are already committed to become adipocytes. Moreover, its ability to detect obesogens acting through a different mechanism than *PPAR $\gamma$*  activation differs between sources and lots (Kassotis et al. 2021, 2022). The commercial availability of MSCs offers a promising alternative to the conventional preadipocyte models. The multipotent nature of MSCs allows the assessment of adipocyte lineage commitment, as well as improved evaluation of adipocyte differentiation (Kassotis et al. 2022; Legler et al. 2020).

Additionally, most studies use 2D monolayer cultures. However, 2D-grown adipocytes have substantial differences in morphology, size, and transcriptional profiles when compared to *in vivo* adipose tissue (Klingelhutz et al. 2018). Recent studies in 3D set-ups have shown 3D adipogenesis models to be more representative of *in vivo* conditions with improved adipocyte differentiation through transcriptional profiling (Klingelhutz et al. 2018; Muller et al. 2019; Shen et al. 2021). These studies underscore the need for multi-omic profiling (transcriptomics, metabolomics, and lipidomics) next to functional readouts in the model for valuable insights into the biology of adipocyte differentiation.

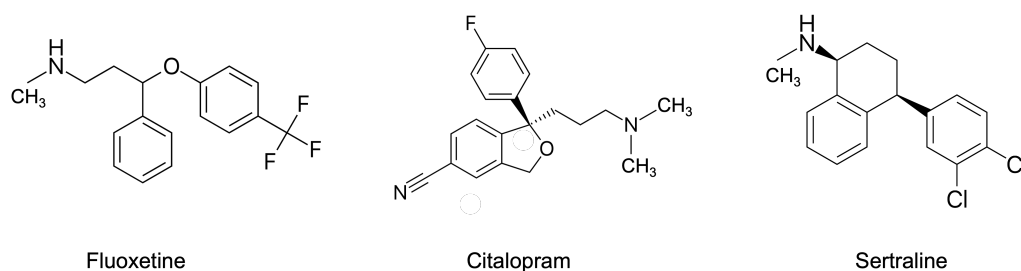
### **2.3. Selective Serotonin Reuptake Inhibitors (SSRIs)**

Major Depressive Disorder (MDD) is characterized by a persistent state of feeling depressed for a minimum of two weeks and patients usually lose interest or pleasure in most activities (Barrera, Torres, and Muñoz 2007). Moreover, when MDD is accompanied by other medical conditions, it leads to a higher burden of disease, diminishing the prognosis of the coexisting medical conditions, prospects for effective treatment, and the quality of life (Belmaker and Agam 2008).

The change towards more isolated lifestyles provided by societal changes has resulted in a rise in depression rates, which in turn increases the prescription of antidepressants. According to the Centers for Disease Control and Prevention, antidepressants consistently rank among the most prescribed medications in the US (Brody and Gu 2020). However, the profit of such extensive prescription is often debated, especially among sensitive populations such as children, adolescents, and pregnant women (Gill et al. 2020; Molenaar et al. 2020; Pariente et al. 2016).

The primary therapeutic indication for antidepressants is the management of MDD. Available antidepressants exhibit a diverse range of chemicals, categorized into subgroups based on their chemical structure and molecular targets. Among these, selective serotonin reuptake inhibitors (SSRIs) represent a group of agents that mainly act on the serotonin transporter (SERT). SSRIs are relatively newer drugs, the first member fluoxetine was marketed in the US in 1988. Since then, they rapidly became the most popular antidepressants in the market due to their high selectivity for the SERT while lacking interactions with histamine, acetylcholine, and  $\alpha$ -adrenoceptors observed with tricyclic antidepressants (TCAs) (Serretti and Kato 2008).

Currently, there are six SSRIs, and the SSRI class has become one of the most used medications worldwide. Members such as fluoxetine, citalopram (CIT), and sertraline (SER) (Figure 2.1) are considered to be the first choice of treatment for moderate to severe MDD. Beyond MDD, SSRIs find applications in other mood disorders including anxiety, post-traumatic stress disorder, obsessive-compulsive disorder, panic disorder, and bulimia. (Serretti and Kato 2008). SSRIs' popularity comes from their user-friendly nature, safety in overdose, relative tolerability, ready availability, and broad use in multiple conditions.



**Figure 2.5** Structures of selective serotonin reuptake inhibitors (SSRIs); fluoxetine, citalopram, and sertraline.

CIT and SER are two of the most prescribed SSRIs (Gill et al. 2020). They have a lipophilic nature, characterized by the log P values of 3.76, and 5.15 for CIT and SER, respectively (Hinkovska-Galcheva et al. 2021). CIT (daily dose 20-60 mg) exhibits rapid absorption from the gastrointestinal tract upon oral administration, typically reaching maximum plasma concentration ( $C_{max}$ ) within 1–4 hours (Wu et al. 2020). Whereas SER (daily dose 20-200 mg), is slowly absorbed, and reaches  $C_{max}$  within 4–8 hours (De Vane et al. 2002). In clinical studies, human steady-state plasma concentrations (SSC) are reported between 40-300 ng/mL (0.12-0.92  $\mu$ M) (Baumann 1996; Gutierrez and Abramowitz 2000; Pollock 2001) for CIT, and 20-200 ng/mL (0.065-0.65  $\mu$ M) (Ronfeld, Tremaine, and Wilner 1997; De Vane et al. 2002), for SER. Notably, around 80 % of CIT is bound to plasma proteins, while the ratio for SER is between 95-99 %. However, they both exhibit long half-lives, from 25 up to 33 hours, attributed to extensive distribution into various organs and tissues, reflected by a large volume distribution of 12-16 L/kg, and 20 L/kg for CIT and SER, respectively (Baumann 1992; De Vane et al. 2002). Finally, a linear correlation exists between CIT and SER within orally administered dose and plasma concentrations at steady state (Baumann 1992; De Vane et al. 2002).

Their advantages aside, CIT and SER's undesired effects on the reproductive system are well-known, including sexual dysfunction, reported in 30-60 % of patients undergoing SSRI treatment. Studies report issues like reduced libido or arousal, and delayed orgasm. These sexual effects tend to continue as long as the patient is on the antidepressant treatment but may improve over time (Gregorian Jr et al. 2002). Another epidemiological study highlighted alterations in hormone levels, including

decreased testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH) levels, and increased prolactin hormone levels in adult men receiving SSRI therapy (Safarinejad 2008).

The mechanism underlying these effects is studied both *in vitro* and *in vivo*. Aromatase (aka. estrogen synthase), is a key enzyme in the process of steroid hormone synthesis (steroidogenesis) responsible for estrogen synthesis. SSRIs were shown to inhibit aromatase activity in two microsome-based *in vitro* assays, disrupting steroidogenesis in the H295R human adrenocortical adenocarcinoma cell line (Hansen et al. 2017; Jacobsen et al. 2015). The estrogenic activity of fluoxetine has been reported both *in vivo* and *in vitro* (Müller et al. 2012). In a similar *in vivo* study, SER and escitalopram (racemic enantiomer of CIT) did not impact the uterus weight of immature rats, suggesting an absence of *in vivo* estrogenic activity as observed with fluoxetine (Montagnini et al. 2013). However, SER has more recently been shown to decrease steroidogenesis in the testis and adrenal glands of male rats, concurrently leading to reduced sperm count and motility *in vivo* (Atli et al. 2017; Munkboel et al. 2018).

Recent evidence suggests a link between long-term SSRI treatment and weight gain in adults, including two of the most prescribed SSRIs, CIT and SER (Arterburn et al. 2016; Blumenthal et al. 2014; Gafoor et al. 2018; Gill et al. 2020; Uguz et al. 2015). Studies report an increase in weight with CIT, ranging from 1.69 kg ( $\geq 4$  months follow-up) to 2.68 kg (24 months follow-up) (Blumenthal et al. 2014; Serretti and Mandelli 2010), while SER was associated with a weight increase, following long-term treatment, ranging from 1.0 kg (9 months follow-up) to 4.76 kg (24 months follow-up) as (Blumenthal et al. 2014; Serretti and Mandelli 2010). Additionally, there are reports on altered lipid profiles such as increased serum triglyceride levels with CIT (an average increase of 18.89 mg/dL,  $p=0.001$ ) and total cholesterol levels with SER (an average increase of 3.85 mg/dL,  $p=0.027$ ) in female patients after 4 months follow-up (Beyazyüz et al. 2013). Yet, the mechanism of the metabolic effects of CIT and SER are poorly understood, and as these pharmaceuticals might contribute to the worldwide increase in obesity, research to these mechanisms is timely.

Considering all the reviewed information, the hypothesis of this thesis was that the SSRIs, CIT and SER, might have obesogenic effects possibly through an endocrine mechanism. Moreover, given their widespread use across diverse populations, including pregnant women, these drugs could potentially contribute to the escalating trends of obesity and metabolic disorders worldwide.

In line with our hypothesis, the objectives of this thesis were:

- 1) Set up and characterize a 3D model with MSCs to enhance the human relevance of the *in vitro* adipogenesis model.
- 2) Investigate the effects of CIT and SER on adipocyte differentiation using human MSCs and murine 3T3-L1 cells.
- 3) Perform mechanistic analyses to elucidate the mode of action of the SSRIs:
  - a. Protein and gene expression analyses: RNA-seq and QPCR analyses in MSCs, in both 2D and 3D models. Western blot analysis in 3T3-L1 cells.
  - b. Functional analyses in MSCs (2D) including the evaluation of lysosomal and phospholipid-inducing effects of the SSRIs.
  - c. Evaluation of receptor-mediated effects of SSRIs using responsive cell lines for various nuclear receptors and ER-dependent proliferation assay. Exploration of SSRIs' effects on the aromatase enzyme with the cell-free aromatase inhibition assay.

### 3. Materials and Methods

#### 3.1. Materials

##### 3.1.1. Chemicals

|                                                   |                                                 |
|---------------------------------------------------|-------------------------------------------------|
| BSA                                               | A3912 (Sigma Aldrich, Germany)                  |
| $\beta$ -ME                                       | M6250 (Sigma Aldrich, Germany)                  |
| Cell Culture Lysis Reagent                        | E1531 (Promega, Leiden, NL)                     |
| CYP19-MFC High-throughput Inhibitor Screening Kit | 459520 (Corning, USA)                           |
| High-Capacity cDNA Reverse Transcription Kit      | 4368814 (Applied Biosystems, USA)               |
| NucleoSpin RNA QPCR Kit                           | 740955.50 (Macherey-Nagel, Germany)             |
| RNA Nano LabChip Kit                              | 5067-1511 (Agilent Technologies, Ca, USA)       |
| iQ™ SYBR® Green Supermix                          | 1708886 (Bio-Rad, NL)                           |
| NaOH                                              | S-0899 (Sigma Aldrich, Germany)                 |
| Trypsin (0.25 %)/ EDTA                            | 25200-056 (Gibco, Thermo Fisher Scientific, NL) |
| hMSC-BM                                           | C-12974 (PromoCell GmbH, Germany)               |
| MSC Growth Medium 2                               | C-28009 (PromoCell GmbH, Germany)               |
| MEM $\alpha$                                      | 22561021 (Gibco, Thermo Fisher Scientific, NL)  |
| DMEM                                              | 41966029 (Gibco, Thermo Fisher Scientific, NL)  |
| DMEM/F-12                                         | 31331028 (Gibco, Thermo Fisher Scientific, NL)  |
| Phenol red-free DMEM                              | 31053028 (Gibco, Thermo Fisher Scientific, NL)  |
| Phenol red-free DMEM/F-12                         | 21041025 (Gibco, Thermo Fisher Scientific, NL)  |



|                                                                     |                                                |
|---------------------------------------------------------------------|------------------------------------------------|
| FBS                                                                 | 10270106 (Gibco, Thermo Fisher Scientific, NL) |
| HEPES                                                               | 15630056 (Gibco, Thermo Fisher Scientific, NL) |
| P/S                                                                 | 15140122 (Gibco, Thermo Fisher Scientific, NL) |
| Geneticin (G418) Sulfate                                            | AG-CN2-0030 (Bio-connect, USA)                 |
| Puromycin                                                           | P8833 (Sigma Aldrich, Germany)                 |
| L-Glutamine (200 mM)                                                | 25030 (Gibco, Thermo Fisher Scientific, NL)    |
| Na-Pyr (100 mM)                                                     | 11360039 (Gibco, Thermo Fisher Scientific, NL) |
| MEM-NAA                                                             | 11140050 (Gibco, Thermo Fisher Scientific, NL) |
| IBMX                                                                | 28822-58-4 (Sigma Aldrich, Germany)            |
| Dex                                                                 | D4902 (Sigma Aldrich, Germany)                 |
| Insulin                                                             | 91077C (SAFC, Switzerland)                     |
| PBS                                                                 | BE17-516F (BioWhittaker)                       |
| Coenzyme A                                                          | C4282 (Sigma Aldrich, Germany)                 |
| EDTA                                                                | E0255 (Sigma Aldrich, Germany)                 |
| DTT                                                                 | D9779 (Sigma Aldrich, Germany)                 |
| D-Luciferin                                                         | E1601 (Promega, Leiden, NL)                    |
| $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ | 22766-8 (Sigma Aldrich, Germany)               |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$                           | 63138 (Sigma Aldrich, Germany)                 |
| Tricine                                                             | T5816 (Sigma Aldrich, Germany)                 |
| EtOH, Absolute                                                      | 1.00986 (Sigma Aldrich, Germany)               |
| Isopropyl alcohol                                                   | 34863 (Sigma Aldrich, Germany)                 |

|                             |                                                  |
|-----------------------------|--------------------------------------------------|
| TCA                         | 27240 (Sigma Aldrich, Germany)                   |
| Acetic acid                 | A6283 (Sigma Aldrich, Germany)                   |
| Tris base                   | T1503 (Sigma Aldrich, Germany)                   |
| Protease Inhibitor Cocktail | 635673 (Takara)                                  |
| Skim milk powder            | SKI400.1 (Bioshop, Canada)                       |
| Xylene                      | 28976294 (VWR, The Netherlands)                  |
| Formaldehyde, 37 %          | 33314 (Alfa Aesar, Thermo Fisher Scientific, NL) |
| Eosine Y                    | E4009 (Merck, Germany)                           |
| Hematoxylin                 | H3136 (Merck, Germany)                           |
| Aluminum potassium sulfate  | 237086-100G (Merck, Germany)                     |
| Sodium iodate               | S4007-100G (Merck, Germany)                      |
| DMSO                        | D8418 (Sigma Aldrich, Germany)                   |
| ROSI                        | 122320-73-4 (Sigma Aldrich, Germany)             |
| SER                         | Y0000828 (Sigma Aldrich, Germany)                |
| CIT                         | PHR1640 (Sigma Aldrich, Germany)                 |
| AMIO                        | A8423 (Sigma Aldrich, Germany)                   |
| β-E2                        | E2758 (Sigma Aldrich, Germany)                   |
| TESTO                       | T-1500 (Sigma Aldrich, Germany)                  |
| TBT                         | 1461-22-9 (Sigma Aldrich, Germany)               |
| TPP                         | 241288 (Sigma Aldrich, Germany)                  |
| PFOA                        | 77262 (Sigma Aldrich, Germany)                   |

|                          |                                                     |
|--------------------------|-----------------------------------------------------|
| MEHP                     | 796832 (Sigma Aldrich, Germany)                     |
| DDE                      | 123897 (Sigma Aldrich, Germany)                     |
| TBBPA                    | 11223 (Sigma Aldrich, Germany)                      |
| FLUD                     | 46102 (Sigma Aldrich, Germany)                      |
| ICI 182,780              | 1047 (Tocris Bioscience, MN, USA)                   |
| FLUT                     | F9397 (Sigma Aldrich, Germany)                      |
| GW7646                   | 10008613 (Cayman Chemical, MI, USA)                 |
| T0070907                 | 2-DXX-89-1 (Toronto Research Chemicals, ON, Canada) |
| AlamarBlue™ Reagent      | DAL1025 (Invitrogen, Thermo Fisher Scientific, NL)  |
| Oil Red O                | O0625 (Sigma Aldrich, Germany)                      |
| Nile Red                 | 72485 (Sigma Aldrich, Germany)                      |
| Bodipy 493/503           | D2191 (Invitrogen, Thermo Fisher Scientific, NL)    |
| Hoechst 33342            | H3570 (Invitrogen, Thermo Fisher Scientific, NL)    |
| LysoTracker Red DND-99   | L7528 (Invitrogen, Thermo Fisher Scientific, NL)    |
| Sulforhodamine B         | 341738 (Sigma Aldrich, Germany)                     |
| KP cryocompound          | K1620 (Immunologic, VWR, NL)                        |
| Entellan mounting medium | 1079610500 (Merck, Germany)                         |

### 3.1.2. Instruments

|                                              |                                              |
|----------------------------------------------|----------------------------------------------|
| CO <sub>2</sub> incubator 150 i              | HERACELL                                     |
| Brightfield microscope AE20 Series           | Motic (China)                                |
| Brightfield microscope CKX41SF (with camera) | Olympus Corporation (Japan)                  |
| Plate reader INFINITE M2000                  | Tecan Austria GmbH                           |
| CellInsight CX5 High-Content screener        | Thermo Fisher Scientific (USA)               |
| Olympus/Evident Spin IXplore SoRa microscope | Olympus Corporation (Japan)                  |
| Agilent 2100 bioanalyzer                     | Agilent Technologies (USA)                   |
| Centrifuge                                   | Eppendorf and Beckman Coulter Inc. (Germany) |
| CFX96 Real-Time System                       | Bio-Rad Laboratories (NL)                    |
| Flow hood                                    | WALDNER mc6                                  |
| Flow cabinet                                 | The Baker Company                            |
| Flow cytometer Accuri C6                     | BD Biosciences (NL)                          |
| Fluorescence microscope                      | Olympus Corporation (Japan)                  |

|                           |                                                |
|---------------------------|------------------------------------------------|
| Freezer (-20°C)           | Bosch                                          |
| Luminometer               | LUMIstar Optima (BMG Labtech)                  |
| NanoDrop                  | Thermo Fischer Scientific (USA)                |
| Western Blot & Gel Imager | Fusion FX7                                     |
| pH meter                  | Metrohm (Germany)                              |
| Pipettors                 | Gilson and Thermo Fischer Scientific (USA)     |
| Refrigerator (+4°C)       | Indesit                                        |
| Scale                     | Mettler Instrumenten (Germany)                 |
| Water purifier            | Milli-Q                                        |
| Water bath                | Gesellschaft für Labortechnik mbH<br>(Germany) |

### **3.1.3. Softwares**

|                          |                                                                     |
|--------------------------|---------------------------------------------------------------------|
| C6 Software v1.0.264.21  | BD Accuri (NL)                                                      |
| GraphPad Prism v9.0      | GraphPad (CA, USA)                                                  |
| Tecan i-control v3.9.1.0 | Tecan Austria GmbH                                                  |
| CellProfiler v4.2.4      | Broad Institute, Massachusetts Institute of<br>Technology (MA, USA) |

|                                                                        |                                                      |
|------------------------------------------------------------------------|------------------------------------------------------|
| ImageJ v2.3.0/1.53q                                                    | National Institutes of Health (MD, USA)              |
| SeqMonk v1.41                                                          | Babraham institute (UK)                              |
| Cytoscape v3.9.1 with ClueGO (v 2.5.9) and CluePedia (v 1.5.9) plugins | National Institute of General Medical Sciences (USA) |

### 3.1.4. Cell Lines

|                                          |                                                              |
|------------------------------------------|--------------------------------------------------------------|
| 3T3-L1                                   | CL173 (ATCC)                                                 |
| hMSC-BM                                  | C-12974 (PromoCell GmbH. Germany)                            |
| VM7Luc4E2 (ER-Luc)                       | Kindly provided by Dr. Jorke Kamstra, Utrecht University, NL |
| T47D-ARE (AR-Luc)                        | Kindly provided by Dr. Jorke Kamstra, Utrecht University, NL |
| H1G1.1c3 (DR-GFP)                        | Kindly provided by Dr. Jorke Kamstra, Utrecht University, NL |
| HG5LN-PPAR $\gamma$ (PPAR $\gamma$ -Luc) | Kindly provided by Patrick Balaguer, DR2 INSERM, FR          |
| HG5LN-PPAR $\alpha$ (PPAR $\alpha$ -Luc) | Kindly provided by Patrick Balaguer, DR2 INSERM, FR          |

### **3.1.5. Preparation of the Solutions Used in *In Vitro* Assays**

#### **3.1.5.1. Stock Solutions for Test Chemicals**

Stock solutions for test and reference chemicals were prepared in DMSO at 1000x higher concentrations than test concentrations and stored at -20°C. For the exposures, stocks were diluted 1:1000 in the medium of the cell line used in the assay, reducing the final DMSO concentration to 0.1 % to avoid cytotoxicity.

#### **3.1.5.2. Stock Solutions for Compounds in Differentiation Medium**

IBMX, Dex, and insulin were used to induce adipocyte differentiation, for both 3T3-L1 cells and MSCs.

10 mM Dex stock was prepared in DMSO, and 10.6 mg/mL insulin solution was prepared in 0.02 N HCl. Dex and insulin stocks were aliquoted and stored at -20 °C prior to exposures. 0.1 M IBMX solution in 0.5 N KOH was prepared fresh on the first exposure day of each experiment.

#### **3.1.5.3. Fixation and Staining Solutions for the Differentiation Assays**

For fixating both 3T3-L1 cells and MSCs, a 3.7 % formaldehyde solution was prepared by diluting 37 % formaldehyde 1:10 in PBS. Fixation and staining steps were done at room temperature (unless indicated differently).

For the staining of 3T3-L1 cells, 5 % (w/v) Oil Red O stock was prepared in isopropyl alcohol and stored at -20 °C. Prior to staining Oil Red O stock was diluted 6:10 in distilled water.

For the staining of MSCs, 1 mg/mL Nile Red and 1 mg/mL Bodipy stock was prepared in DMSO and stored at -20 °C. Hoechst 33342 solution (10 mg/mL) was purchased from Thermo Fischer and stored at 4 °C. Prior to staining Nile Red or Bodipy was diluted 1:1000, and Hoechst 1:10000 in PBS (in the same staining solution). 1 mM LysoTracker stock was stored at -20 °C and diluted 1:10000 in culture medium for live staining.

#### **3.1.5.4. Solutions for Hematoxylin & Eosin Staining**

Hematoxylin solution was prepared by dissolving 0.2 g Hematoxylin and 10 g aluminum potassium sulfate in 200 mL distilled water. After Hematoxylin was

completely dissolved, 40 mg sodium iodate and 4 mL acetic acid were added to the solution. Subsequently, the solution was heated up to just before boiling temperature and allowed to cool before filtering. The Hematoxylin solution was stored away from light at room temperature until further use.

Eosin Y solution was prepared by dissolving 0.5 g Eosin Y in 50 mL distilled water. Subsequently, 20 mL of filtered Eosin Y stock solution and 10 drops of acetic acid were added to 150 mL of 80 % EtOH. Eosin Y solution was stored away from light at room temperature until further use.

#### **3.1.5.5. Luciferase Solution for the ER-Luc and AR-Luc Assays**

Luciferase solution was prepared by dissolving Tricine (final conc. 20 mM); 130 mg  $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$ ; 165 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; EDTA (final conc. 0.1 mM); DTT (final conc. 33.3 mM) in approximately 200 mL Milli-Q water in the water bath. After these compounds were dissolved, the solution was cooled down to room temperature, 50 mg Coenzyme A; 37.4 mg D-Luciferin; and 80.2 mg ATP were added, and the final volume was adjusted to 250 mL with MilliQ water. Aliquots of 50 mL were stored at -80 °C, in aluminum foil-covered glass bottles until further use.

#### **3.1.5.6. Alamar Blue Solution for Cytotoxicity Assay**

For the cytotoxicity assay, Alamar Blue Reagent was diluted to 5 % v/v in PBS (*e.g.*, 5 mL Alamar Blue was added to 95 mL PBS). The solution was prepared fresh for all experiments in the desired amount and used non-sterile.

#### **3.1.5.7. Solutions for Sulforhodamine B Assay**

10 % (w/v) TCA solution was prepared in distilled water for the fixation of MCF-7 cells. 1 % (v/v) acidic acid solution was prepared in distilled water. 4 mg/mL Sulforhodamine B solution was prepared in 1 % acidic acid and stored at room temperature. 10 mM tris base solution was prepared in distilled water, and pH was adjusted to 10.7 using 1 M NaOH, prior to use.



## **3.2.Methods**

### **3.2.1. Adipogenesis Assays**

#### **3.2.1.1. Human Mesenchymal Stem Cell (MSC) Model**

##### ***Cell Culture and Differentiation***

Multipotent human MSCs isolated from the bone marrow of a 61-year-old Caucasian male donor were purchased from PromoCell (Germany) at passage 2 and expanded in the recommended media (MSC growth medium 2 with 10 % Supplement Mix (PromoCell)) according to manufacturer's instructions. Cells were subcultured once after thawing at 70–80 % confluency (passage 3), and frozen in MSC growth medium 2 containing 5 % DMSO (passage 4).

For all experiments, a new batch of MSCs was thawed and maintained in MEM $\alpha$  supplemented with 15 % FBS, 1 % P/S, and 2 % HEPES (culture medium) in 75 cm<sup>2</sup> cell culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>. Cells were subcultured once after thawing at 70–80 % confluency (passage 5). After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of 2–3x10<sup>4</sup> cells. All experiments were performed at passage 6. MSCs were differentiated into mature adipocytes in 3D and 2D, using similar protocols but with adjustments for the different models.

##### ***3D Model***

MSCs were seeded at a density of 5x10<sup>3</sup> cells per well in 0.2 mL culture medium in the inner wells of 96 well ultra-low attachment (ULA) plates (Corning® 7007) (outer rows were filled with PBS). After seeding, ULA plates were centrifuged at 150 g for 2 min and incubated for 2 days at 37 °C and 5 % CO<sub>2</sub> for spheroid formation. Spheroid formation was confirmed under the microscope 2 days after seeding, differentiation was induced by replacing half of the medium (0.1 mL) with 2x differentiation medium, consisting of culture medium supplemented with 1 mM IBMX, 0.2  $\mu$ M Dex, and 10  $\mu$ g/mL insulin (Table 3.1). Fresh IBMX stock was prepared on the first exposure day

for each experiment, Dex and insulin were used from stock solutions prepared in advance.

Differentiation medium was prepared 2x concentrated for the initial exposure, followed by refreshments with 1x differentiation medium. Half of the medium was refreshed subsequently with 1x differentiation medium (culture medium supplemented with 0.5 mM IBMX, 0.1  $\mu$ M Dex, and 5  $\mu$ g/mL insulin) every 3 to 4 days (after initial exposure) (Fig. 3.1.a).

**Table 3.1** Concentrations of the different compounds in differentiation medium.

| <i>Name</i> | <i>Stock conc.</i>       | <i>Final conc. (1x)</i> | <i>Final conc. (2x)</i> |
|-------------|--------------------------|-------------------------|-------------------------|
| Dex         | 10 mM in DMSO            | 0.1 $\mu$ M             | 0.2 $\mu$ M             |
| Insulin     | 10.6 mg/mL in 0.02 N HCl | 5 $\mu$ g/mL            | 10 $\mu$ g/mL           |
| IBMX        | 0.1 M in 0.5 N KOH       | 0.5 mM                  | 1 mM                    |

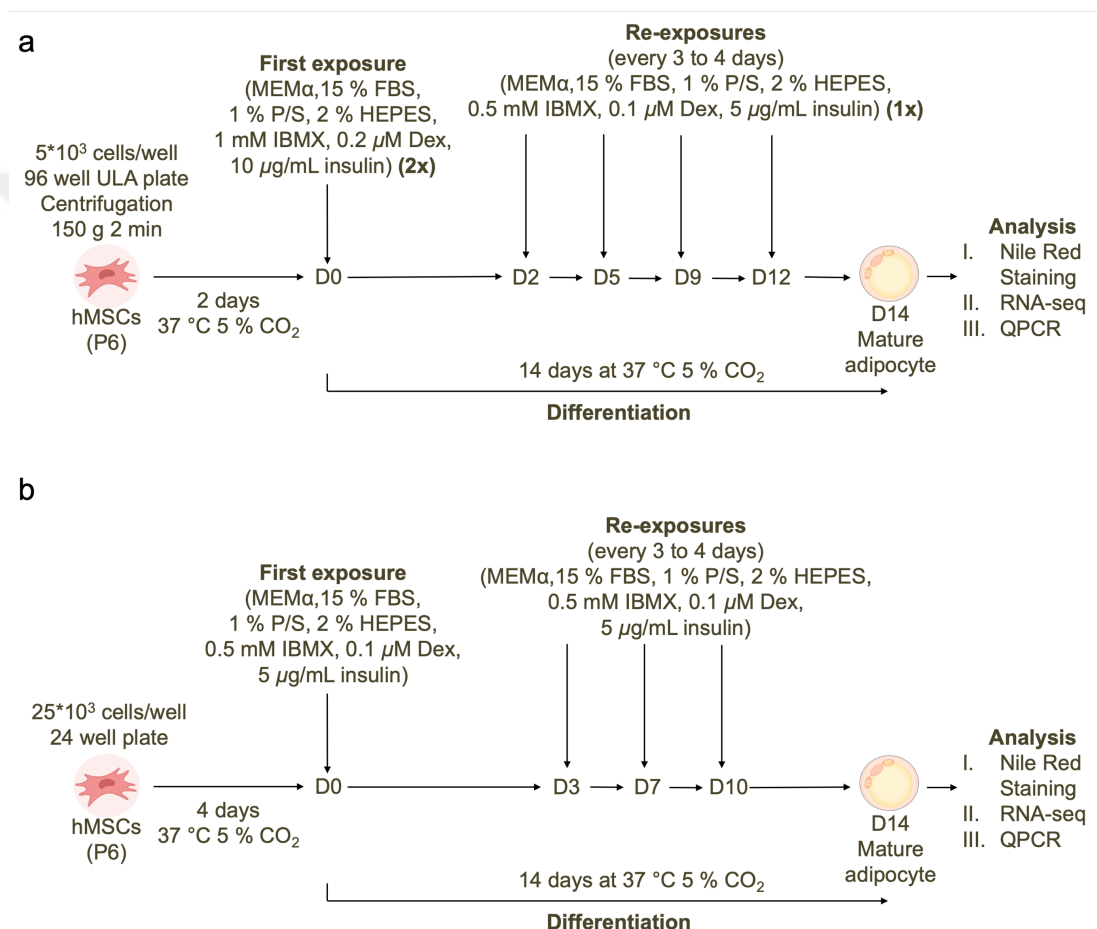
## **2D Model**

MSCs were seeded at a density of  $25 \times 10^3$  cells per well in 1 mL culture medium in 24 well plates (Greiner Bio-One) and incubated for 4 days at 37 °C and 5 % CO<sub>2</sub>. Adipocyte differentiation was induced at 100 % confluency, 4 days after seeding, by replacing the entire culture medium with 1x differentiation medium (culture medium supplemented with 0.5 mM IBMX, 0.1  $\mu$ M Dex, and 5  $\mu$ g/mL insulin) (Table 3.1). Differentiation medium with the assigned exposures was refreshed subsequently every 3 to 4 days for 14 days (after initial exposure) (Fig. 3.1.b).

DMSO (0.1 %) was used as vehicle control for all adipogenesis experiments. ROSI at 0.5  $\mu$ M was used as positive control in 2D and 3D, as well as inducing MSC differentiation for the 2D mature adipocyte experiments. Cells were exposed to 6 up to 8 concentrations of SER and CIT in a range around reported SSCs. 20-200 ng/mL (0.065-0.65  $\mu$ M) (Gupta and Dziurdzy 1994; Ronfeld et al. 1997; De Vane et al. 2002), and 40-300 ng/mL (0.12-0.92  $\mu$ M) (Baumann 1996; Dufour et al. 1987; Gutierrez and Abramowitz 2000; Rochat et al. 1998) for SER and CIT, respectively. During method development of the 3D model, a battery of obesogens was tested in parallel experiments in 2D and 3D. The selection was based on the known adipogenic activity of the chemicals. ROSI and TBT were tested from 6 up to 9 concentrations (ROSI:

0.0001–0.5  $\mu\text{M}$ , and TBT: 0.00001–0.3  $\mu\text{M}$ ), while the obesogens BPA, TBBPA, FLUD, PFOA, MEHP, TOCP, TPP, and *p,p'*-DDE were tested at 4 concentrations (3.75, 7.5, 15, and 30  $\mu\text{M}$ ).

3 independent experiments were performed for all adipogenesis assays (2D and 3D), each experiment consisted of a maximum of 6 (3D) or 3 (2D) plate replicates.

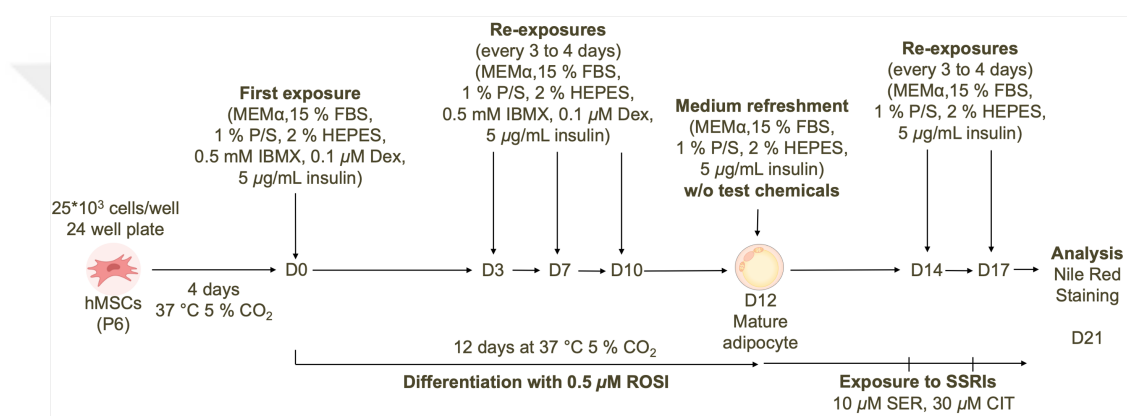


**Figure 3.1** Schematic representation of human mesenchymal stem cell (MSC) differentiation under (a) 3D, and (b) 2D conditions. The first exposure, day 0 (D0), indicates the beginning and re-exposures indicate medium refreshments with the assigned exposures.

For the mature adipocyte experiments the 2D model was used with adjustments to the protocol. MSCs were differentiated in 2D, as previously described, with 0.5  $\mu\text{M}$  ROSI for 12 days. On day 12, differentiation medium was replaced with culture medium

containing only 5  $\mu\text{g/mL}$  insulin (insulin medium) and incubated for 2 days. After 2 days, insulin medium was refreshed and mature adipocytes were exposed to the SSRIs, SER at 10  $\mu\text{M}$  and CIT at 30  $\mu\text{M}$ . SSRI exposure was repeated after 3 days. Subsequently, fixated cells were stained for analysis on day 21 (after initial exposure) (Fig. 3.2).

Differences in methodologies for the experimental setup for 3D, 2D and the mature adipocyte assays are summarized in Table 3.2.



**Figure 3.2** Schematic representation of the mature adipocyte assay. First exposure, day 0 (D0), indicates the beginning and re-exposures (D3, D7, and D10) indicate medium refreshments with 0.5  $\mu\text{M}$  rosiglitazone (ROSI). D12 shows medium refreshment with culture medium containing only 5  $\mu\text{g/mL}$  insulin, and re-exposures (D14 and D17) indicate medium refreshments with 10  $\mu\text{M}$  sertraline (SER) and 30  $\mu\text{M}$  citalopram (CIT).

**Table 3.2** Differences in methodologies for MSC differentiation in 3D, 2D and the mature adipocyte assays.

|                                                       | <i>3D</i>                                     | <i>2D</i>                                                      | <i>2D Mature adipocytes</i>                         |
|-------------------------------------------------------|-----------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------|
| Cell culture plates                                   | 96 well ultra-low attachment (ULA, Corning)   | 24 well (Greiner Bio-One)                                      | 24 well (Greiner Bio-One)                           |
| Cell density per well                                 | 5x10 <sup>3</sup>                             | 25x10 <sup>3</sup>                                             | 25x10 <sup>3</sup>                                  |
| Seeding volume (mL)                                   | 0.2                                           | 1                                                              | 1                                                   |
| Time between seeding and first exposure               | 2 days                                        | 4 days                                                         | 4 days                                              |
| Differentiation medium conc. (for the first exposure) | 2x                                            | 1x                                                             | 1x                                                  |
| Total amount of exposures                             | 5                                             | 4                                                              | 6                                                   |
| Re-exposure procedure                                 | Replacement of 50 % medium, every 3 to 4 days | Replacement of the entire medium, every 3 to 4 days            | Replacement of the entire medium, every 3 to 4 days |
| Reference chemical                                    | ROSI (0.5 $\mu$ M)                            | ROSI (0.5 $\mu$ M)                                             | ROSI (0.5 $\mu$ M)                                  |
| Assay duration                                        | 14 days                                       | 14 days                                                        | 21 days                                             |
| Analysis method                                       | High-throughput imaging<br>RNA-seq<br>QPCR    | Fluorescence plate reader<br>Flow cytometry<br>RNA-seq<br>QPCR | Fluorescence plate reader                           |

### ***Assessment of Adipocyte Differentiation***

After differentiation, cells were fixated and stained (except for flow cytometry analysis) with Nile Red and Hoechst, for intracellular lipids and cell nuclei, respectively. Nile Red signal was measured and quantified using different analysis methods (Table 3.3). An increase in Nile Red intensity was used as a measure for enhanced adipocyte differentiation.

Cytotoxicity was determined via visual inspections and in some cases, Hoechst staining, since due to heterogeneity of the cell population cytotoxicity assays were not possible.

For the measurement of 3D spheroids, 50 % medium was removed from the wells at every step, therefore 2x concentrated solutions were prepared for the fixation and staining (Table 3.3). Cells were fixated with 7.4 % formaldehyde (37 %) solution in PBS for 30-min, rinsed 2 times with 0.1 mL PBS (again by removal of 50 % fluid for the washing steps) and subsequently stained for intracellular lipids with 1  $\mu\text{g/mL}$  Nile Red and 5  $\mu\text{g/mL}$  Hoechst for 1.5h, the given concentrations are the final concentrations in the well. After staining, cells were rinsed 2 times with PBS and left with 0.2 mL PBS per well. Images were taken with a high-content microscope (CellInsight™ CX5 High-Content Screening (HCS) Platform (Thermo Scientific)) of each spheroid at 10x magnification. Nile Red was imaged in the FITC channel (Ex 482/35, Em 536/40) and Hoechst in the DAPI channel (Ex 377/50, Em 447/60) and analyzed using CellProfiler software (v4.2.4) (Stirling et al. 2021)

For the measurement of 2D adipocytes, Nile Red fluorescence was either measured by a plate reader or via flow cytometry. A similar fixation and staining procedure was performed for the fluorescence plate reader, but by refreshment of the entire medium at every step, therefore preparing 1x concentrated solutions in PBS (Table 3.3). Cells were first fixed with 3.7 % formaldehyde solution in PBS for 30-min, rinsed 2 times with 0.5 mL PBS, and subsequently stained with 10  $\mu\text{g/mL}$  Nile Red and 0.5  $\mu\text{g/mL}$  Hoechst for 1.5h. After staining, cells were rinsed 2 times with 0.5 mL PBS and left with 1 mL PBS per well. Nile Red was measured at Ex/Em 485/590 nm for neutral lipids, Ex/Em 585/645 nm for phospholipids, and Hoechst at Ex/Em 355/460 nm for cell nuclei with a plate reader (Tecan, Infinite M2000).

**Table 3.3** Fixation and staining concentrations for 2D and 3D.

|                          | 3D                             | 2D                             |
|--------------------------|--------------------------------|--------------------------------|
| <i>Fixation (30-min)</i> | <i>Final conc. in PBS (2x)</i> | <i>Final conc. in PBS (1x)</i> |
| Formaldehyde             | 7.4 %                          | 3.7 %                          |
| <i>Staining (1.5h)</i>   |                                |                                |
| Nile Red                 | 1 $\mu\text{g/mL}$             | 10 $\mu\text{g/mL}$            |
| Hoechst                  | 5 $\mu\text{g/mL}$             | 0.5 $\mu\text{g/mL}$           |

For flow cytometry analysis, the exposure medium was aspirated and cells were rinsed with 0.5 mL PBS, and trypsinized (0.25 mL) for 10-min. After cell detachment was confirmed under the microscope, 0.75 mL freshly prepared buffer (Milli-Q water containing 1 % BSA, 2 mM EDTA, and 0.025  $\mu\text{g/mL}$  Nile Red) was added to each well, cells were resuspended by pipetting up and down, and incubated for at least 10-min. Half of the plate was measured at a time, cells were resuspended again to prevent clogging of the machine. Neutral and phospholipid accumulation was assessed using the Accuri C6 flow cytometer (BD Biosciences, NL). Analysis was performed by using the positive control ROSI as a basis for the gating strategy. The first gating (P1) was for separating cells from debris, based on SSC-A (side scatter-area)/FSC-A (forward scatter-area). Cells were further sorted according to lipid profiles (P2) based on Nile Red intensity, optical filters FL2 (Em 585/40) and FL3 (Em > 670) were used for neutral and phospholipids, respectively.

### ***Brightfield and Fluorescence Microscopy***

Brightfield microscopy on H&E-stained sections of spheroids was used to examine the morphology of 3D spheroids. Spheroids were fixed as described above. For cryosectioning, spheroids that were exposed to DMSO and ROSI (n = 6) were transferred from the 96 well plate to microcentrifuge tubes. Excess PBS was removed and 0.2  $\mu\text{L}$  KP cryocompound was added to the tube. A cryomold was prepared by freezing two layers of clear colored KP cryocompound at -20°C and the KP cryocompound containing spheroids was transferred to the cryomold. A new layer of yellow-colored KP cryocompound was added on top to embed the spheroid. Leica CM3050s cryostat was used to cut 10  $\mu\text{m}$  slices of the spheroids. The slices were dried overnight on SuperFrost Plus, Adhesion Slides. For H&E staining, slides were incubated in distilled water for 30s; hematoxylin solution for 2-min; eosin solution for

10s and rinsed with 95 % ethanol for 30s. Followed by two-times 30s incubation in absolute EtOH and 2-min incubation in xylene. All slides were cover slipped with Entellan mounting medium and dried overnight. Pictures were taken with the Olympus BX60 WF microscope under 20x magnification.

For fluorescence imaging, stained adipocytes from 2D and 3D assays (see above) were used. Hoechst was imaged in the DAPI channel (Ex 377/50, Em 447/60), Nile Red was imaged in the FITC channel (Ex 482/35, Em 536/40) for neutral lipids, and Texas Red channel for phospholipids (2D). 3D spheroids were imaged with Olympus/Evident Spin IXplore SoRa microscope under 20x magnification. 2D adipocytes were imaged with Leica DM IL LED microscope using LAS X software, under 20x magnification.

### ***Assessment of Lysosomes***

SSRIs' effects on lysosomes during differentiation were studied using a cell-permeable fluorescent dye that stains acidic compartments, LysoTracker Red DND-99. Amiodarone (AMIO), a broad-spectrum antiarrhythmic drug and a known inducer of phospholipidosis (Reasor et al. 2006), was used for inducing phospholipids and lysosomes. 3 independent experiments were performed for lysosome assessment, with 3 plate replicates.

For the experiments, MSCs were differentiated in 2D as previously described. After 14 days of differentiation, cells were live stained with 100 nM LysoTracker Red for lysosome assessment, 10  $\mu$ g/mL Bodipy 493/503 for neutral lipids, and 0.5  $\mu$ g/mL Hoechst for cell nuclei. The staining solution was prepared in culture medium, according to the manufacturer's instructions, cells were incubated for 1.5h at 37 °C and 5 % CO<sub>2</sub>. After staining, cells were rinsed 2 times with 0.5 mL PBS and left with 1 mL PBS per well. LysoTracker Red signal was measured using a fluorescence plate reader (Tecan, Infinite M2000) at Ex/Em 565/599 nm, Bodipy at Ex/Em 486/526 nm, and Hoechst at Ex/Em 355/460 nm (Table 3.4).



**Table 3.4** Staining concentrations for MSCs (2D).

| <i>Fluorescent dye</i>             | <i>Final conc. in PBS</i> | <i>Ex/Em (nm)</i> |
|------------------------------------|---------------------------|-------------------|
| Nile Red ( <u>neutral lipids</u> ) | 10 $\mu\text{g/mL}$       | 485/590           |
| Nile Red ( <u>phospholipids</u> )  |                           | 585/645           |
| Bodipy 493/503                     | 10 $\mu\text{g/mL}$       | 486/526           |
| LysoTracker                        | 100 nM                    | 565/599           |
| Hoechst                            | 0.5 $\mu\text{g/mL}$      | 355/460           |

Ex/Em: Excitation/emission wavelengths for the fluorescence plate reader.

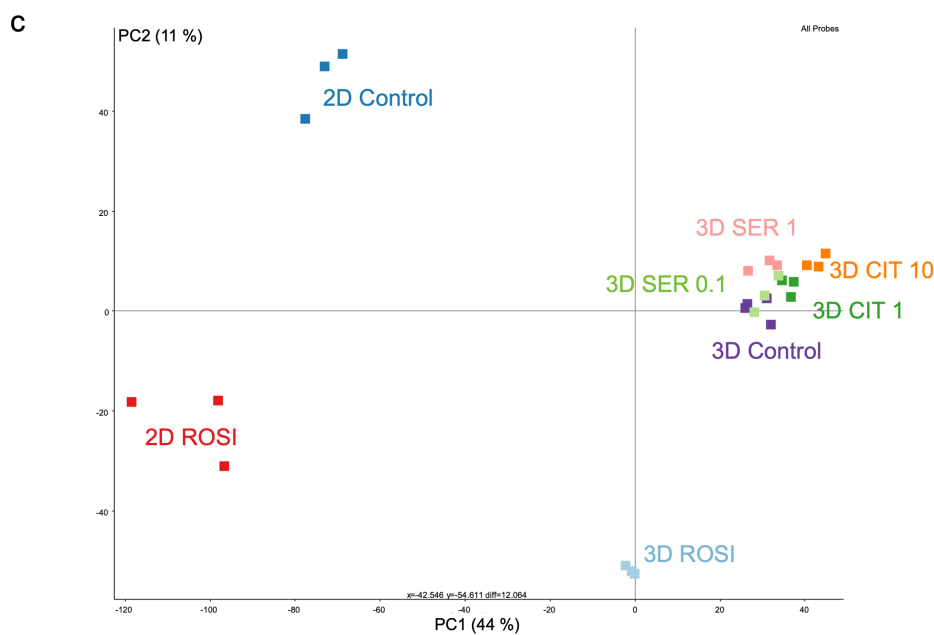
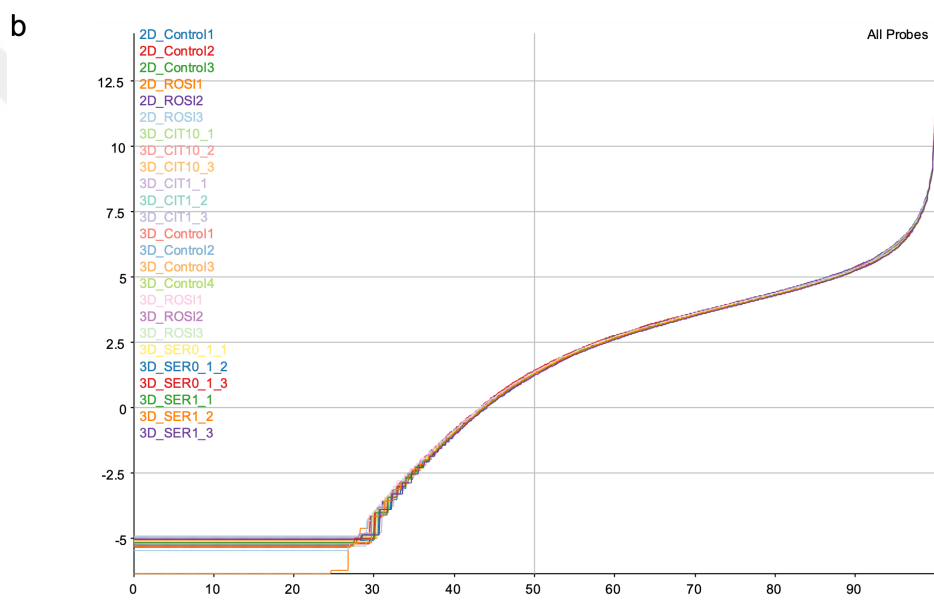
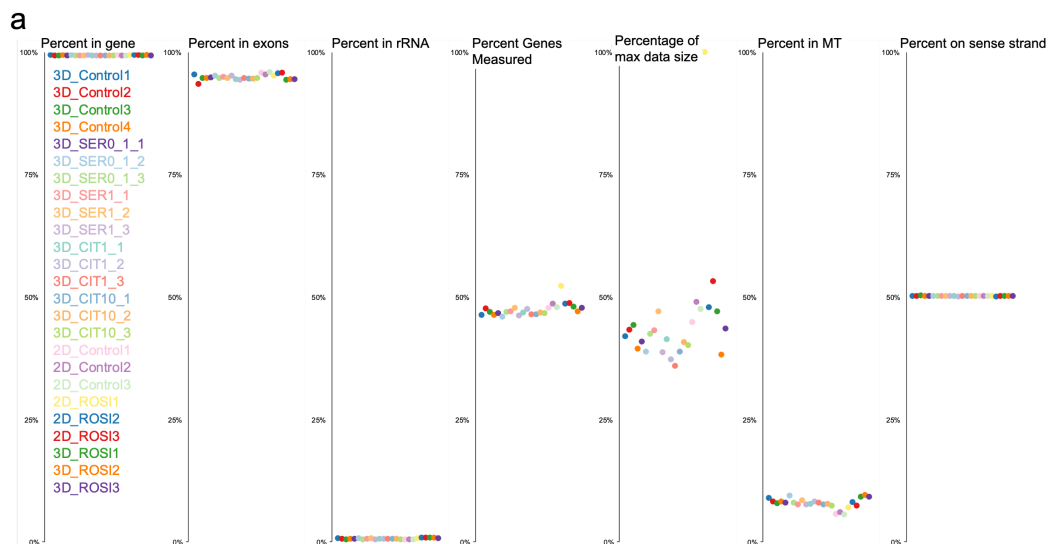
### ***RNA-sequencing***

RNA-sequencing (RNA-seq) was performed in 2D and 3D differentiated MSCs to compare transcriptional profiles of the two models, as well as to clarify the mechanism behind SSRIs' effects. To compare the two models, cells were treated with either control (0.1 % DMSO) or positive control (0.1  $\mu\text{M}$  ROSI) in parallel 2D and 3D protocols. For the assessment of SSRIs, CIT and SER were tested at their NOEC and LOEC in the 3D model (CIT: 1, 10  $\mu\text{M}$ , SER: 0.1, 1  $\mu\text{M}$ ). 3 independent experiments were performed for both 2D and 3D, with one extra control for 3D.

Following the adipogenesis assays, total RNA was isolated and purified using the NucleoSpin® RNA extraction kit, according to the manufacturer's instructions, from one confluent well of a 24 well plate or 10–20 spheroids pooled together for 2D and 3D, respectively. RNA integrity number (RIN) was determined with Agilent 2100 Bioanalyzer using RNA Nano LabChip Kit. All samples were found to be of acceptable quality for sequencing (RIN > 9.0) and sent to Novogene (UK) for sequencing by poly-A capture, library preparation, and analysis on the Illumina Novaseq using 150bp paired end sequencing. Raw fastq files were adapter trimmed using trim\_galore (v0.4.5, Babraham Institute, UK) under standard parameters. STAR aligner (v2.5.4 b) was used to align and map sequences to the homo sapiens genome (GRCh38\_v102; [https:// www.ensembl.org](https://www.ensembl.org)) (Dobin et al. 2013). After alignment, the generated BAM files were loaded to SeqMonk (v1.41, Babraham Institute) and mRNAs were quantified using the built-in mRNA seq pipeline. Data quality plots were generated, and all data was found to be of acceptable quality (Fig. 3.3.a-b). The clustering of samples based on model and treatment is shown in Fig. 3.3.c. Normalization of the read counts and differential expression was conducted using the

Deseq2 method (Love et al. 2014). Normalized counts for all genes for the different treatments were clustered using ClustVis and visualized using a PCA plot (Metsalu and Vilo 2015). The RNA-seq data has been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev, and Lash 2002) and is accessible through GEO Series accession number GSE242103 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE242103>).

For GSEA, DEGs were scored based on their p-value and shrunken  $\log_2$  fold changes ( $\log(\text{p-value}) \times -\log_2(\text{FC})$ ). The scored genes were loaded to Webgestalt ([www.webgestalt.org](http://www.webgestalt.org)), and GSEA was performed using the KEGG pathway and GO databases. GSEA parameters were adjusted to only include pathways containing a minimum of 5 and a maximum of 200 genes per pathway. The enrichment statistic (p) was set to 0 and the FDR threshold to 1 to obtain the full pathway lists. Subsequently, pathways were selected based on FDR values where at least one of the individual treatments exhibited a significant change ( $\text{FDR} < 0.05$ ). For 2D vs 3D model comparisons, pathways that were significantly altered with ROSI treatment were used. NES were used for a more accurate comparison of the enriched pathways with different gene numbers (Xie, Jauhari, and Mora 2021). Heatmaps with NES of significantly altered pathways were generated using ClustVis in which at least one condition was significantly affected. Row centering and row scaling were disabled to visualize true NES scores. Functional networks were constructed in Cytoscape (v 3.9.1) using the ClueGO (v 2.5.9) and CluePedia (v 1.5.9) plugins (Bindea et al. 2009). Significant KEGG and GO pathways of each condition were imported to perform a Preselected Functions analysis using the KEGG and GO biological processes ontologies (v 16-05-2023). GO term fusion was enabled to prevent duplicate pathways. For further exploration of the RNA-seq data, DEGs were selected for either one of the treatments in 3D (n=3848) and clustered using the k-means method (Cluster 3.0) (Koch et al. 2018). Silhouette score was used to determine the optimal number of clusters and evaluate the quality of clustering. DEG lists of clusters were imported to Webgestalt for ORA with KEGG pathways, using the complete gene list as a reference set (the rest of the parameters were kept the same). Representative pathways and DEGs, based on significance (FDR), were selected from each cluster and presented as a heatmap using GraphPad (v9.0).



**Figure 3.3** RNA quality plots. (a) RNA QC plot. Sequences shown on different features of the human genome. (b) Cumulative distribution plot. The distribution of log2 RPM values is shown from low to highly expressed genes in all samples. (c) PCA analysis of all measured genes, showing the first and second PC based on correlation clustering. Control: DMSO, ROSI: Rosiglitazone, CIT: Citalopram, SER: Sertraline.

### ***QPCR Analysis***

Expression of selected adipogenic genes was evaluated by QPCR analysis to confirm the results of RNA-seq. To investigate the differences in gene expression, cells were differentiated with vehicle control 0.1 % DMSO, positive control ROSI (0.1  $\mu$ M), TBT (0.01 nM), and the obesogens BPA, TBBPA, FLUD, PFOA, MEHP, TOCP, TPP, and *p,p'*-DDE (15  $\mu$ M) in parallel via 2D or 3D protocols. All exposures were repeated in 3 independent experiments.

For the assessment of SSRIs, CIT and SER were tested at their NOEC and LOEC in the 3D model (CIT: 1, 10  $\mu$ M, SER: 0.1, 1  $\mu$ M). Exposures were repeated in 2 independent experiments.

Following the adipogenesis assays, RNA isolation and purification was performed as previously described. RNA amount of 2D samples was measured using Nanodrop-2000 Spectrophotometer (NanoDrop Technologies, DE, USA). Equal amounts of RNA were converted into cDNA with the high-capacity cDNA RT kit (Applied Biosystems, Grand Island, NY) according to the manufacturer's recommendations. The cDNA was diluted 10-times with sterile water. For 3D, RNA was directly converted into cDNA due to low amounts of RNA (below the detection limit of Nanodrop), and diluted 5x. For 3D, RNA yield was qualitatively assessed by checking the Cq value of Beta Actin during QPCR.

QPCR analysis was performed on a CFX96 (Bio-Rad Laboratories, NL) in technical duplicates for each sample in a reaction volume of 10  $\mu$ L with 5  $\mu$ L iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix, 250 nM of forward and reverse primers, 2.5  $\mu$ L of diluted cDNA, and nuclease-free water. QPCR protocol was 3-min denaturation at 95°C, followed by 40 cycles of 15s at 95°C and 45s at 60°C. After the run a melting curve was generated

from 65 to 95 °C (Table 3.5). All primers have been tested for efficiency (by serial dilutions) and specificity (by melting curve). After testing various reference genes (Primer sequences for reference genes and genes of interest are provided in Table 3.6), Beta Actin and Nono were selected to calculate normalized gene expression using the  $\Delta\Delta C_q$  method. Differential gene expression was calculated as log2 fold changes compared to vehicle control. Data was clustered and presented as a heatmap using ClustVis (Metsalu and Vilo 2015) with rows and columns clustered using Euclidean distance and Ward linkage.

**Table 3.5** QPCR protocol.

| <i>Setting</i>                      | <i>Temperature (°C)</i>     | <i>Duration</i> |
|-------------------------------------|-----------------------------|-----------------|
| Step 1 (denaturation)               | 95                          | 3 min           |
| Step 2                              | 95                          | 15 sec          |
| Step 3                              | 60                          | 45 sec          |
| Step 4 (repetitions of steps 2 & 3) |                             | 40 min          |
| Step 5 (melting curve)              | 65 to 95 in steps of 0.5 °C | 5 sec per step  |

**Table 3.6** Primer sequences for QPCR analysis.

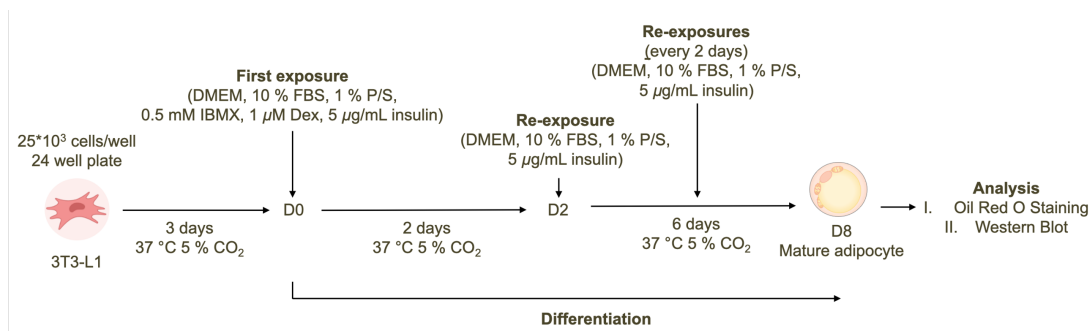
| <i>Name</i>                           | <i>Gene</i>                    | <i>Species</i> | <i>Forward Primer</i> | <i>Reverse Primer</i>   |
|---------------------------------------|--------------------------------|----------------|-----------------------|-------------------------|
| PPAR gamma                            | <i>PPAR<math>\gamma</math></i> | Human          | GCGATTCCTTCACTGATAC   | TCAAAGGAGTGGGAGTGGTC    |
| CEBP alpha                            | <i>CEBP<math>\alpha</math></i> | Human          | TATAGGCTGGGCTTCCCCTT  | AGCTTTCTGGTGTGACTCGG    |
| Fatty acid binding protein 4          | <i>FABP4</i>                   | Human          | AAACTGGTGGTGAATGCGT   | GCGAACTTCAGTCCAGGTCA    |
| Adiponectin                           | <i>ADIPOQ</i>                  | Human          | TCCATACCAGAGGGGCTCAG  | GAGTCGTGGTTTCCTGGTCA    |
| Insulin receptor                      | <i>INSR</i>                    | Human          | GGCGATATGGTGATGAGGAGC | CTGTCACGTAGAAATAGGTGGGT |
| Insulin-like growth factor 1 receptor | <i>IGF1R</i>                   | Human          | ACGAGTGGAGAAATCTGCGG  | ATGTGGAGGTAGCCCTCGAT    |
| Beta actin                            | <i>Bactin</i>                  | Human          | GAGCACAGAGCCTCGCC     | TCATCATCCATGGTGAGCTGG   |
| Nono                                  | <i>Nono</i>                    | Human          | TCGGTAGAGGAGAAGTCGAGG | CTCTGCATTTTTGCACCCTCA   |

### 3.2.1.2. 3T3-L1 Preadipocyte Model

#### *Cell Culture and Differentiation*

3T3-L1 mouse fibroblast cell line was purchased from (ATCC), expanded in DMEM supplemented with 10 % FBS and 1 % P/S in 75 cm<sup>2</sup> cell culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>. Cells were subcultured upon reaching 70–80 % confluency. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and 3 mL cell suspension was transferred onto 12 mL culture medium (1:5) in a 75 cm<sup>2</sup> flask. All experiments were performed up to passage 10 for optimal differentiation capacity (Kassotis et al. 2021).

3T3-L1 cells were seeded at a density of  $25 \times 10^3$  cells per well in 1 mL culture medium in 24 well plates (Greiner Bio-One) and incubated for 3 days at 37 °C and 5 % CO<sub>2</sub>. Adipocyte differentiation was induced at 100 % confluency, 3 days after seeding, by replacing the entire culture medium with differentiation medium (culture medium supplemented with 0.5 mM IBMX, 1  $\mu$ M Dex, and 5  $\mu$ g/mL insulin) with test chemicals. After a 2-day incubation period, the differentiation medium was replaced with culture medium supplemented with only 5  $\mu$ g/mL insulin (insulin medium), and the assigned exposures. The insulin medium with the assigned exposures was refreshed subsequently every two days for 6 days (day 8 after initial exposure) (Fig. 3.4).



**Figure 3.4** Schematic representation of 3T3-L1 differentiation. First exposure, day 0 (D0), indicates the beginning and re-exposures indicate medium refreshments with the assigned exposures.

0.1 % DMSO was used as vehicle control, and ROSI at 1  $\mu$ M as positive control for all adipogenesis assays. Cells were exposed to 6 up to 8 concentrations of SER and CIT in a range around reported steady-state plasma concentrations (SSCs). 20-200 ng/mL (0.065-0.65  $\mu$ M) (Gupta and Dziurdzy 1994; Ronfeld et al. 1997; De Vane et al. 2002), and 40-300 ng/mL (0.12-0.92  $\mu$ M) (Baumann 1996; Dufour et al. 1987; Gutierrez and Abramowitz 2000; Rochat et al. 1998) for SER and CIT, respectively.

#### ***Assessment of Adipocyte Differentiation***

After differentiation, cells were first fixated with 0.5 mL of 3.7 % formaldehyde (37 %) for 15 min, and rinsed with 0.5 mL PBS. Subsequently, intracellular lipids were stained with 200  $\mu$ L Oil Red O for 30 min. After staining, cells were rinsed with distilled water and imaged under a microscope under 4X magnification. Oil Red O absorbance was measured by a plate reader (Thermo Scientific VarioScan) at 518 nm. An increase in Oil Red O intensity was used as a measure for enhanced adipocyte differentiation.

Cytotoxicity was determined via visual inspections since due to the heterogeneity of the cell population cytotoxicity assays were not possible.

#### ***Western Blot Analysis***

The expressions of selected adipogenic proteins (PPAR $\gamma$ , C/EBP $\alpha$ , SREBP1, LPL, and FAS) were investigated by western blot analysis. DMSO (0.1 %) was used as vehicle control, ROSI at 1  $\mu$ M as positive control, while CIT and SER were tested at 30 and



10  $\mu$ M, respectively. 3 independent experiments were performed for western blot analysis.

Following the adipogenesis assays, the exposure medium was aspirated, cells were rinsed with 0.5 mL PBS and trypsinized (0.5 mL). After cell detachment was confirmed under the microscope cells from each confluent well of a 24-well plate were collected in separate Eppendorf tubes and centrifuged at 2400 g at 4 °C for 5 min. Cell pellet was suspended by pipetting up and down in 80  $\mu$ L lysis buffer and subsequently lysed with an ultrasonic homogenizer. The homogenate was centrifuged at 18000 g at 4 °C for 10 min and the supernatant was collected separately from each tube. The total protein concentration of the samples was calculated, 15  $\mu$ g protein homogenates were diluted with loading buffer (leameli buffer) and denaturated at 95°C for 5 min. Samples were loaded on a 20 % SDS polyacrylamide gel (3.76 mL 1 M tris base (ph 8.8), 4 mL 30 % acrylamide, 50  $\mu$ L 20 % SDS, 50  $\mu$ L 10 % APS, 5 $\mu$ L TEMED was added to 2.2 mL distilled water, making the total volume 10 mL (for two gels)). After electrophoresis, proteins on the gel were transferred onto the PVDF membrane and blocked for 1h with 5 % non-fat skim milk in TBS-T. Membranes were left to incubate with primary antibodies overnight at 4 °C. The next day, membranes were washed 4 times with TBS-T and incubated with goat-anti-rabbit/mouse IgG-HRP conjugate (Proteintech) for 45 min. Protein bands on membranes were visualized by chemiluminescence ECL substrate using Fusion FX7 instrument. Protein bands were normalized to Beta Actin bands using Image J software and protein expression was calculated as log2 fold changes and presented as a heatmap using GraphPad Prism (v9.0).

### **3.2.2. Receptor Binding and Transactivation Assays**

#### ***PPAR $\gamma$ -Luc Assay***

HG5LN-PPAR $\gamma$  cell line was used for the PPAR $\gamma$ -Luc assay. HG5LN-PPAR $\gamma$  cell line was created by the stable transfection of HeLa cell line (epithelial cells isolated from the cervical adenocarcinoma of a 31-year-old female patient) with a responsive luciferase reporter gene plasmid (pGAL4-GR- puro) to induce luciferase activity upon activation of PPARs by a ligand (Seimandi et al. 2005).

PPAR $\gamma$ -Luc cells were maintained in DMEM/F-12 medium supplemented with 10 % FBS, 1 % P/S, G418 (final conc. 1 mg/mL) and Puromycin (final conc. 0.5  $\mu$ g/mL) (culture medium) in 75 cm<sup>2</sup> cell culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>.

Cells were subcultured upon reaching 80-90 % confluency, every 3 to 4 days. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of 2.5-3.5x10<sup>6</sup> cells.

Cells were used up to passage 50 for the assays. 3 independent experiments were performed for all reporter assays (agonism and antagonism), each experiment consisted of 3 plate replicates. SSRIs were tested at 7 concentrations (SER: 0.01–10  $\mu$ M and CIT: 0.04–30  $\mu$ M) to obtain efficient concentration-response curves.

HG5LN-PPAR $\gamma$  cells were seeded at a density of 5x10<sup>4</sup> cells per well in 150  $\mu$ L culture medium in white opaque 96-well plates (655083, Greiner Bio-One) and left to attach for 24h at 37 °C and 5 % CO<sub>2</sub>. After 24h, medium was replaced with 200  $\mu$ L assay medium (phenol red-free DMEM/F-12 supplemented with 5 % DCC FBS, 1 % P/S) containing vehicle control (0.1 % DMSO) or test chemicals in the absence or presence of reference agonist, ROSI. EC<sub>50</sub> of the reference agonist was determined as 10 nM in the agonism assay and was used to activate the receptor in the antagonism assay.

After 24h incubation (37 °C and 5 % CO<sub>2</sub>), the medium was replaced with 50  $\mu$ L luminescence medium (assay medium containing 0.3 mM D-luciferin (3 %)) per well. After 20-min incubation (room temperature) luciferase activity was measured in intact cells using a luminometer (LUMIstar Optima, BMG Labtech). Agonist activities are calculated as a percentage of the maximal luciferase activity induced by ROSI. Antagonist activities are calculated as a percentage of the luciferase activity induced by 10 nM ROSI.

#### ***PPAR $\alpha$ -Luc Assay***

HG5LN-PPAR $\alpha$  cell line was used for the PPAR $\alpha$ -Luc assay. HG5LN-PPAR $\alpha$  cell line was created by the stable transfection of HeLa cell line (epithelial cells isolated from the cervical adenocarcinoma of a 31-year-old female patient) with a responsive

luciferase reporter gene plasmid (pGAL4-GR- puro) to induce luciferase activity upon activation of PPARs by a ligand (Seimandi et al. 2005).

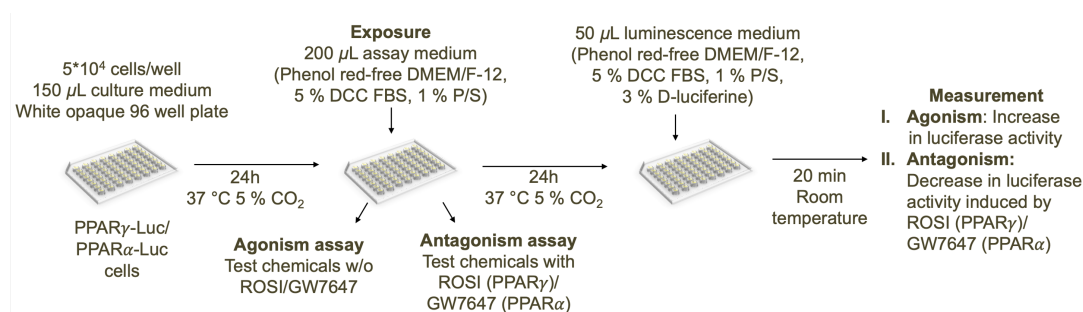
PPAR $\alpha$ -Luc cells were maintained in DMEM/F-12 medium supplemented with 10 % FBS, 1 % P/S, G418 (final conc. 1 mg/mL) and Puromycin (final conc. 0.5  $\mu$ g/mL) (culture medium) in 75 cm<sup>2</sup> cell culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>.

Cells were subcultured upon reaching 80-90 % confluency, every 3 to 4 days. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of 2.5-3.5x10<sup>6</sup> cells.

Cells were used up to passage 50 for the assays. 3 independent experiments were performed for all reporter assays (agonism and antagonism), each experiment consisted of 3 plate replicates. SSRIs were tested at 7 concentrations (SER: 0.01–10  $\mu$ M and CIT: 0.04–30  $\mu$ M) to obtain efficient concentration-response curves.

HG5LN-PPAR $\alpha$  cells were seeded at a density of 5x10<sup>4</sup> cells per well in 150  $\mu$ L culture medium in white opaque 96-well plates (655083, Greiner Bio-One) and left to attach for 24h at 37 °C and 5 % CO<sub>2</sub>. After 24h, the medium was replaced with 200  $\mu$ L assay medium (assay medium composition was the same as the PPAR $\gamma$ -Luc assay, see above) containing vehicle control (0.1 % DMSO) or test chemicals in the absence or presence of reference agonist, GW7647. EC50 of the reference agonist was determined as 31.7 nM in the agonism assay and was used to activate the receptor in the antagonism assay.

After 24h incubation (37 °C and 5 % CO<sub>2</sub>), the medium was replaced with 50  $\mu$ L luminescence medium (assay medium containing 0.3 mM D-luciferin (3 %)) per well. After 20-min incubation (room temperature) luciferase activity was measured in intact cells using a luminometer (LUMIstar Optima, BMG Labtech). Agonist activities are calculated as a percentage of the maximal luciferase activity induced by GW7647. Antagonist activities are calculated as a percentage of the luciferase activity induced by 31.7 nM GW7647. Fig. 3.5 shows the schematic representation of the PPAR $\gamma$ -Luc and PPAR $\alpha$ -Luc assays.



**Figure 3.5** Schematic representation of the PPAR $\gamma$ -Luc and PPAR $\alpha$ -Luc assays. PPAR: Peroxisome proliferator-activated receptor, ROSI: Rosiglitazone.

### ***DR-GFP Assay***

H1G1.1c3 mouse hepatoma cell line was created by stable transfection of mouse hepatoma (Hepal1c7) cells with the AhR-EGFP reporter plasmid pGreen 1, to produce a fluorescent protein (GFP) upon activation of the AhR by a ligand (Nagy et al. 2002). Another widely used term for the AhR is the dioxin receptor (DR) because of its well-known affinity for dioxin compounds. Thereby, this assay will be referred to as the DR-GFP assay, and the term DR will be employed from this point onward in this thesis to prevent confusion for the reader.

H1G1.1c3 cells were maintained in DMEM supplemented with 10 % FBS and 1 % P/S (culture medium) in 75 cm<sup>2</sup> tissue culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>.

Cells were subcultured upon reaching 80-90 % confluency, every 3 to 4 days. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of 2.5-3.5x10<sup>6</sup> cells.

Cells were used up to passage 30 for the assays. 3 independent experiments were performed for all reporter assays (agonism and antagonism), each experiment consisted of 3 plate replicates. SSRIs were tested at 7 concentrations (SER: 0.01–10  $\mu$ M and CIT: 0.04–30  $\mu$ M) to obtain efficient concentration-response curves.

For the DR-GFP assay H1G1.1c3 cells were seeded at a density of  $3 \times 10^4$  cells per well in 100  $\mu$ L culture medium in the inner wells of transparent 96-well plates (Greiner Bio-One) and the outer wells were filled with PBS. Cells were left to attach for 24h at 37°C and 5 % CO<sub>2</sub>. After 24h, medium was replaced with 200  $\mu$ L culture medium containing vehicle control (0.1 % DMSO) or test chemicals in the absence or presence of reference agonist, TCDD. EC<sub>50</sub> of the reference agonist was determined as 37 pM in the agonism assay, and was used to activate the receptor in the antagonism assay.

After 6h and 24h incubation with test chemicals, the fluorescence signal was measured at Ex/Em 485/530 nm with a plate reader (Tecan, Infinite M2000) in intact cells without the removal of the medium. Agonist activities are calculated as percentages of the maximum fluorescence signal induced by TCDD. Antagonist activities are calculated as percentages of the fluorescence signal induced by 37 pM TCDD.

#### ***ER-Luc Assay***

VM7Luc4E2 cell line was used for the ER-Luc assay. Cells were derived from the solid primary tissue from a patient with Stage III, very poorly differentiated ovarian adenocarcinoma and stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ERE) to produce luciferase activity upon activation of the ER by a ligand (Rogers and Denison 2000).

VM7Luc4E2 cells were maintained in DMEM supplemented with 10 % FBS and 1 % P/S (culture medium) in 75 cm<sup>2</sup> tissue culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>.

Cells were subcultured upon reaching 80-90 % confluency, every 3 to 4 days. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of  $2.5\text{--}3.5 \times 10^6$  cells.

Cells were used up to passage 30 for the assays. 3 independent experiments were performed for all reporter assays (agonism and antagonism), each experiment consisted of 3 plate replicates. SSRIs were tested at 7 concentrations (SER: 0.01–10  $\mu$ M and CIT: 0.04–30  $\mu$ M) to obtain efficient concentration-response curves.

Prior to ER-Luc assay, the culture medium of VM7Luc4E2 cells was replaced with assay medium and maintained for one medium change (four days) before seeding for the experiments. The assay medium consisted of phenol red-free DMEM supplemented with 10 % DCC FBS, 1 % P/S, 1 % L-Glutamine (stock conc. 200 mM) and 1 % Na-pyr (stock conc. 100 mM).

Cells were seeded at a density of  $4 \times 10^4$  cells per well in 200  $\mu$ L assay medium in the inner wells of transparent 96-well plates (Greiner Bio-One) and the outer wells were filled with PBS. Cells were left to attach for 48h at 37 °C and 5 % CO<sub>2</sub>. After 48h, the medium was replaced with 200  $\mu$ L assay medium containing vehicle control (0.1 % DMSO) or test chemicals in the absence or presence of reference agonist,  $\beta$ -E2. EC50 of the reference agonist was determined as 3 pM in the agonism assay and was used to activate the receptor in the antagonism assay.

After 24h incubation with test chemicals, the medium was removed, cells were washed with 200  $\mu$ L PBS and subsequently lysed with 25  $\mu$ L cell culture lysis reagent for 30 min on a plate shaker, at room temperature. Luciferase activity was measured using a luminometer (LUMIstar Optima, BMG Labtech) by the addition of 100  $\mu$ L luciferase solution directly to each well prior to measurement, followed by 100  $\mu$ L 0.2 M NaOH injection to stop luminescence, preventing interference with the subsequent well. Agonist activities are calculated as percentages of the maximum luciferase activity induced by  $\beta$ -E2. Antagonist activities are calculated as percentages of the luciferase activity induced by 3 pM  $\beta$ -E2.

### ***AR-Luc Assay***

T47D-ARE cell line was used for the AR-Luc assay. Cells were isolated from a pleural effusion obtained from a 54-year-old female patient with infiltrating ductal carcinoma of the breast and stably transfected with an androgen-responsive luciferase reporter gene plasmid (pGudLuc7ARE) to produce luciferase activity upon activation of the AR by a ligand (Blankvoort et al. 2001).

T47D-ARE cells were maintained in DMEM supplemented with 10 % FBS and 1 % P/S (culture medium) in 75 cm<sup>2</sup> tissue culture flasks in a humidified incubator at 37 °C and 5 % CO<sub>2</sub>.

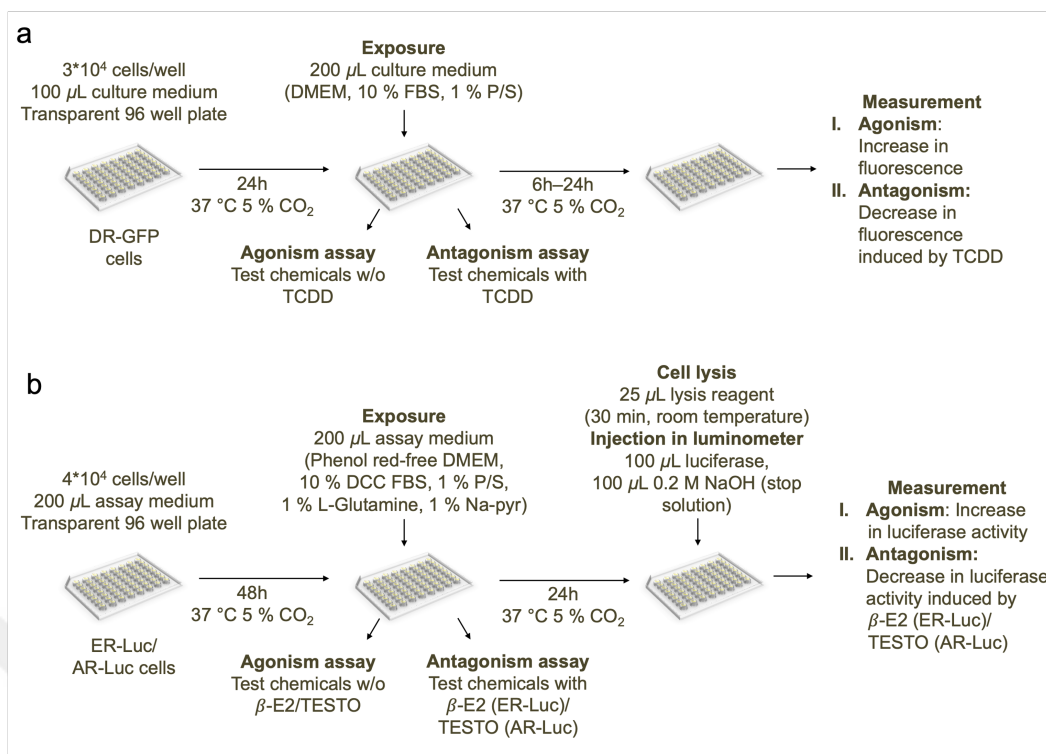
Cells were subcultured upon reaching 80-90 % confluency, every 3 to 4 days. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and transferred into a 75 cm<sup>2</sup> flask at a density of 2.5-3.5x10<sup>6</sup> cells.

Cells were used up to passage 30 for the assays. 3 independent experiments were performed for all reporter assays (agonism and antagonism), each experiment consisted of 3 plate replicates. SSRIs were tested at 7 concentrations (SER: 0.01–10  $\mu$ M and CIT: 0.04–30  $\mu$ M) to obtain efficient concentration-response curves.

Similar to ER-Luc assay, culture medium of T47D-ARE cells was replaced with assay medium prior to AR-Luc assay and maintained for one medium change (four days) before seeding for the experiments. Assay medium composition was the same as the ER-Luc assay (see above).

Cells were seeded at a density of 4x10<sup>4</sup> cells per well in 200  $\mu$ L assay medium in the inner wells of transparent 96-well plates (Greiner Bio-One) and the outer wells were filled with PBS. Cells were left to attach for 48h at 37°C and 5 % CO<sub>2</sub>. After 48h, medium was replaced with 200  $\mu$ L assay medium containing vehicle control (0.1 % DMSO) or test chemicals in the absence or presence of reference agonist, TESTO. EC<sub>50</sub> of the reference agonist was determined as 20 nM in the agonism assay and was used to activate the receptor in the antagonism assay.

After 24h incubation with test chemicals, medium was removed, cells were washed with 200  $\mu$ L PBS and subsequently lysed with 25  $\mu$ L cell culture lysis reagent for 30 min on a plate shaker, at room temperature. Luciferase activity was measured using a luminometer (LUMIstar Optima, BMG Labtech) by the addition of 100  $\mu$ L luciferase solution directly to each well prior to measurement, followed by 100  $\mu$ L 0.2 M NaOH injection to stop luminescence, preventing interference with the subsequent well. Agonist activities are calculated as percentages of the maximum luciferase activity induced by TESTO. Antagonist activities are calculated as percentages of the luciferase activity induced by 20 nM TESTO. Schematic representation of the DR-GFP, ER-Luc, and AR-Luc assays are shown in Fig. 3.6.a-b.

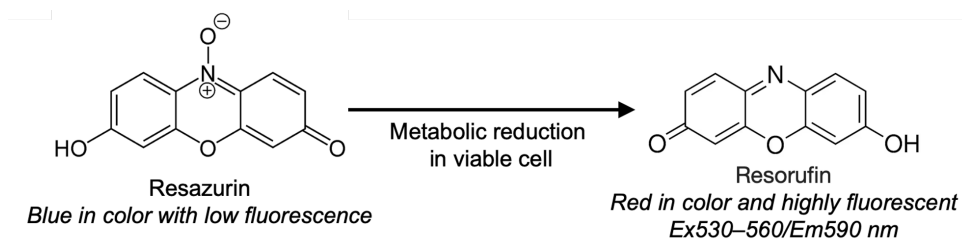


**Figure 3.6** Schematic representation of (a) DR-GFP, (b) ER-Luc and AR-Luc assays.

### *Alamar Blue Assay*

To detect true antagonistic responses of test chemicals that are not due to cytotoxicity, cell viability was determined for the reporter cells (ER-Luc, AR-Luc, DR-GFP, PPAR $\gamma$ -Luc and PPAR $\alpha$ -Luc) via the Alamar Blue assay. Cells were seeded and exposed to test chemicals in the same manner. 24h after exposures cells were washed with 200  $\mu$ L PBS and 100  $\mu$ L Alamar Blue solution (5 % v/v in PBS) was added to each well. Plates were incubated for 45 min at 37 °C and 5 % CO<sub>2</sub>, followed by measurement at Ex/Em 540/590 nm with a fluorescence plate reader (Tecan, Infinite M2000). Metabolic reduction of the resazurin dye to resorufin in live cells was used as a measure of cell viability (Fig. 3.7).





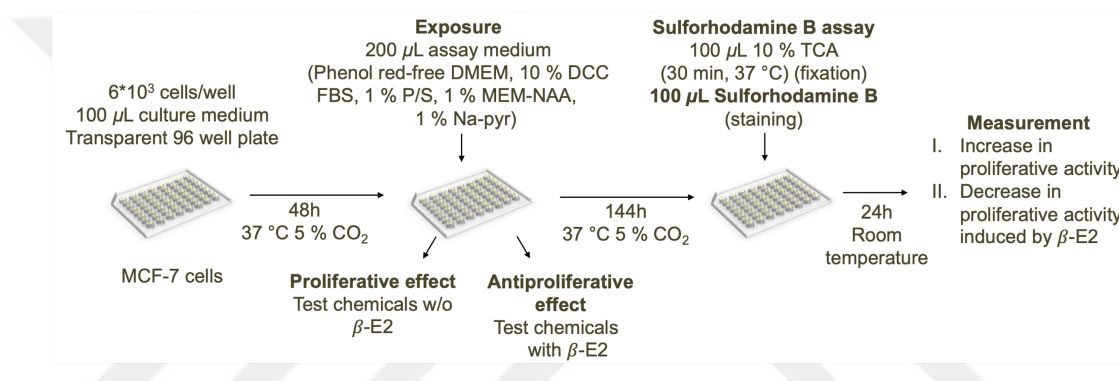
**Figure 3.7** Principle of Alamar Blue assay. Metabolic reduction of resazurin dye in viable cells.

### 3.2.3. ER-dependent Proliferation Assay

MCF-7 is a hormone-dependent human breast cancer cell line, expressing estrogen receptor (ER +) which makes it responsive to estrogens. Therefore, a valuable tool for assessing the estrogenic/anti-estrogenic activity of chemicals (Soto et al. 1995). MCF-7 cells were maintained in DMEM supplemented with 10 % FBS and 1 % P/S in a humidified atmosphere at 37 °C, 5 % CO<sub>2</sub> and passaged upon reaching approximately 80 % confluency. After aspirating the culture medium, cells were washed with prewarmed PBS and detached with trypsin-EDTA. Trypsin was deactivated with 6 mL culture medium. Cells were spun down at 800 rpm for 5 min, resuspended, and 3 mL cell suspension was transferred onto 12 mL culture medium (1:5) in a 75 cm<sup>2</sup> flask. Experiments were performed up to passage 40. 3 independent experiments were performed for both proliferative and antiproliferative activity, each experiment consisted of 4 plate replicates. SSRIs were tested at 8 concentrations (0.001–33 μM) to obtain efficient dose-response curves.

For the proliferation assays MCF-7 cells were seeded at a density of 6x10<sup>3</sup> cells per well in 100 μL culture medium in transparent 96-well plates (Greiner Bio-One). Cells were left to attach for 48h at 37 °C and 5 % CO<sub>2</sub>. After 48h, medium was replaced with 200 μL assay medium (phenol red-free DMEM supplemented with 10 % DCC FBS, 1 % P/S, 1 % MEM-NAA, 1 % Na-pyr) with vehicle control (0.5 % DMSO) or the test chemicals in the absence or presence of reference agonist, β-estradiol (β-E2) (Fig. 3.8).

After 144h incubation with test chemicals, cell proliferation was evaluated with sulforhodamine B (SRB) assay (Vichai and Kirtikara 2006). Medium was aspirated, and cells were fixed with 100  $\mu$ L 10 % TCA solution at 4 °C for 30 min and stained with 100  $\mu$ L SRB solution. Excess dye was washed off with 1 % acetic acid and plates were left to dry at room temperature for 24h. After 24h, stained cells were solubilized with 10 mM tris base solution (pH 10.7), the absorbance was measured at 492 nm on a plate reader (Thermo Scientific Varioscan). Activities are calculated as percentages of the induction with 1 nM  $\beta$ -E2. Cytotoxicity was evaluated via visual inspections.



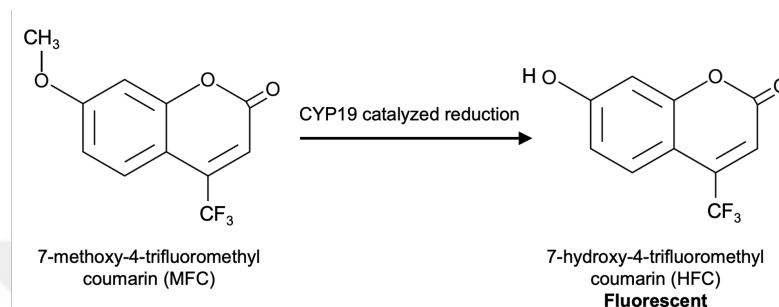
**Figure 3.8** Schematic representation of the estrogen receptor (ER)-proliferation assay with MCF-7 cells.  $\beta$ -E2:  $\beta$ -Estradiol.

### 3.2.4. Aromatase Inhibition Assay

Aromatase inhibitory activity was evaluated by CYP19/MFC High-throughput Screening kit (Corning), according to the manufacturer's instructions. Acetonitrile (ACN) was used as vehicle control, and ketoconazole (KET) at 40  $\mu$ M as reference compound. SSRIs were tested at 8 concentrations (0.015–33  $\mu$ M) to obtain efficient concentration-response curves.

The cell-free assay relies on the aromatase (CYP19) catalyzed reduction of 7-methoxy-4-trifluoromethyl coumarin (MFC) to its fluorescent metabolite 7-hydroxy-4-trifluoromethyl coumarin (HFC) (Fig. 3.9). After incubation of test chemicals with

human recombinant CYP19 and MFC, fluorescence signal was measured at Ex/Em 409/530 nm with a fluorescence plate reader (Thermo Scientific VarioScan). A decrease in fluorescence intensity was used as a measure for CYP19 inhibition. Results are expressed as percent fluorescence compared to vehicle control, ACN.



**Figure 3.9** Aromatase (CYP19) catalyzed reduction of 7-methoxy-4-trifluoromethyl coumarin (MFC) to its fluorescent metabolite, 7-hydroxy-4-trifluoromethyl coumarin (HFC).

### 3.2.5. Data Analysis

In adipogenesis assays, intracellular lipid accumulation was quantified by fluorescence staining, and the intensities for the different stains (see methods) were corrected for cell number (Hoechst intensity, when applicable) and expressed as fold change. In reporter cell line and ER-dependent proliferation assays, results are expressed as percentages of maximum luciferase or fluorescence activity induced by the reference chemical. Aromatase inhibitory activities are expressed as percent fluorescence compared to vehicle control (ACN).

Data from independent experiments are analyzed in GraphPad (v9.0) by two-way ANOVA with treatment and experiment number as independent variables. No main effect or interaction effect was found for replicate experiments. Subsequently, data is averaged over the independent experiments and used for statistical analysis using GraphPad (v9.0) with one-way ANOVA, employing Dunnett's multiple comparisons test, after the Shapiro-Wilk tests for normal distribution analysis (GraphPad (v9.0)).

### ***Benchmark Dose Modelling***

Data from MSC experiments were used for benchmark dose (BMD) modeling via PROAST Web (v70.1, RIVM, NL) (Hardy et al. 2017). Nile Red intensity, as fold change and averaged over the independent experiments, were put in as continuous summary data with standard deviation as dispersion measure. The standard 5 % change was considered not sufficient, therefore critical effect size (CES) was set at 20 % (Norgren et al. 2022), corresponding to a 1.2-fold induction compared to control. Two different families of models were fit to the data (exponential and Hill), Akaike Information Criterion (AIC) was set to two, and model averaging was not employed. The resulting confidence intervals were expressed in terms of BMDL and BMDU, the lower and upper bound of the 90 % confidence interval, respectively.

## 4. Results

### 4.1. Assessment of Adipocyte Differentiation

#### 4.1.1. Human Mesenchymal Stem Cell (MSC) Model

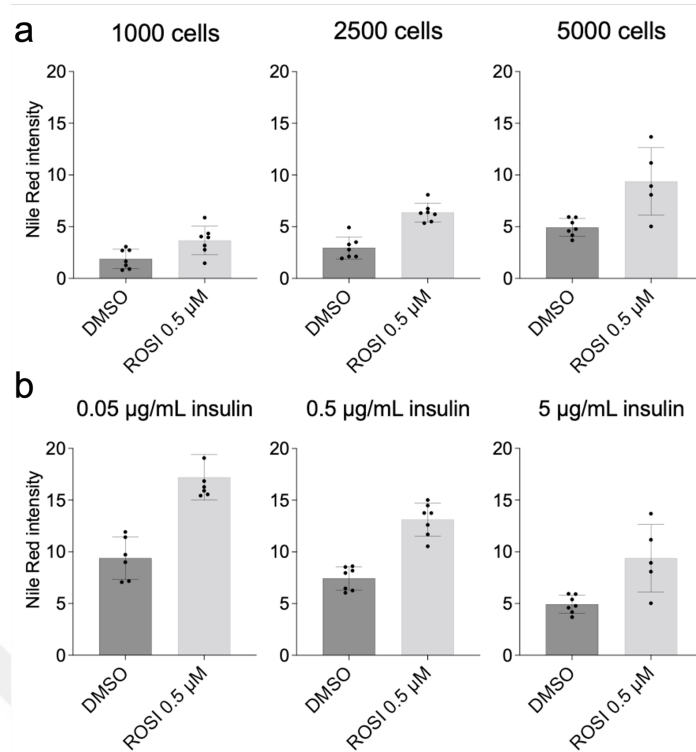
For a comprehensive assessment of SSRIs' adipogenic potential, the effects on adipocyte differentiation were investigated by using MSCs in 2D and 3D models. MSCs provide important advantages in the assessment of obesogens, such as improved assessment of adipocyte commitment and differentiation (Kassotis et al. 2022; Legler et al. 2020).

##### 4.1.1.1. Optimization and Applicability of the 3D Adipogenesis Model

One of the main objectives of this thesis was to set up and characterize a human-relevant *in vitro* 3D adipogenesis model, as 3D models are reported to be more physiologically relevant compared to conventional 2D models in recent studies (Klingelhutz et al. 2018; Shen et al. 2021).

##### *Optimization of 3D Assay Conditions*

First, the 3D assay conditions were optimized for optimal spheroid growth and differentiation. 5000 cells per spheroid was determined to be optimal for spheroid formation and growth, with positive control ROSI showing higher differentiation compared to lower seeding densities of 1000 or 2500 cells per spheroid (Fig. 4.1.a). Additionally, spheroid morphology was more stable with 5000 cells compared to the lower seeding densities. After optimizing seeding density, different concentrations of insulin (0.05, 0.5, 5  $\mu\text{g/mL}$ ) in the differentiation medium were tested to achieve optimal differentiation. The lowest basal differentiation (differentiation with vehicle control, DMSO) was obtained at 5  $\mu\text{g/mL}$  insulin, which led to a higher induction with positive control, ROSI (Fig. 4.1.b). Despite higher differentiation with medium which contained lower insulin, 5  $\mu\text{g/mL}$  insulin was determined to be optimal for 3D differentiation, making the assay more adept at distinguishing potential adipogenic effects.



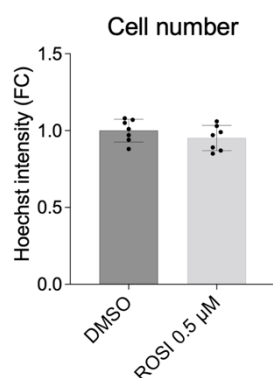
**Figure 4.1** Growth and differentiation of 3D spheroids with (a) seeding densities of 1000, 2500, and 5000 cells/well, and (b) insulin concentrations of 0.05, 0.5, and 5  $\mu$ g/mL. Spheroids were differentiated with control (0.1 % DMSO) or positive control 0.5  $\mu$ M rosiglitazone (ROSI). Bars represent averages of 6 to 8 spheroids per condition, error bars indicate standard deviation (GraphPad Prism, v9.0).

### ***Morphology of 3D Spheroids***

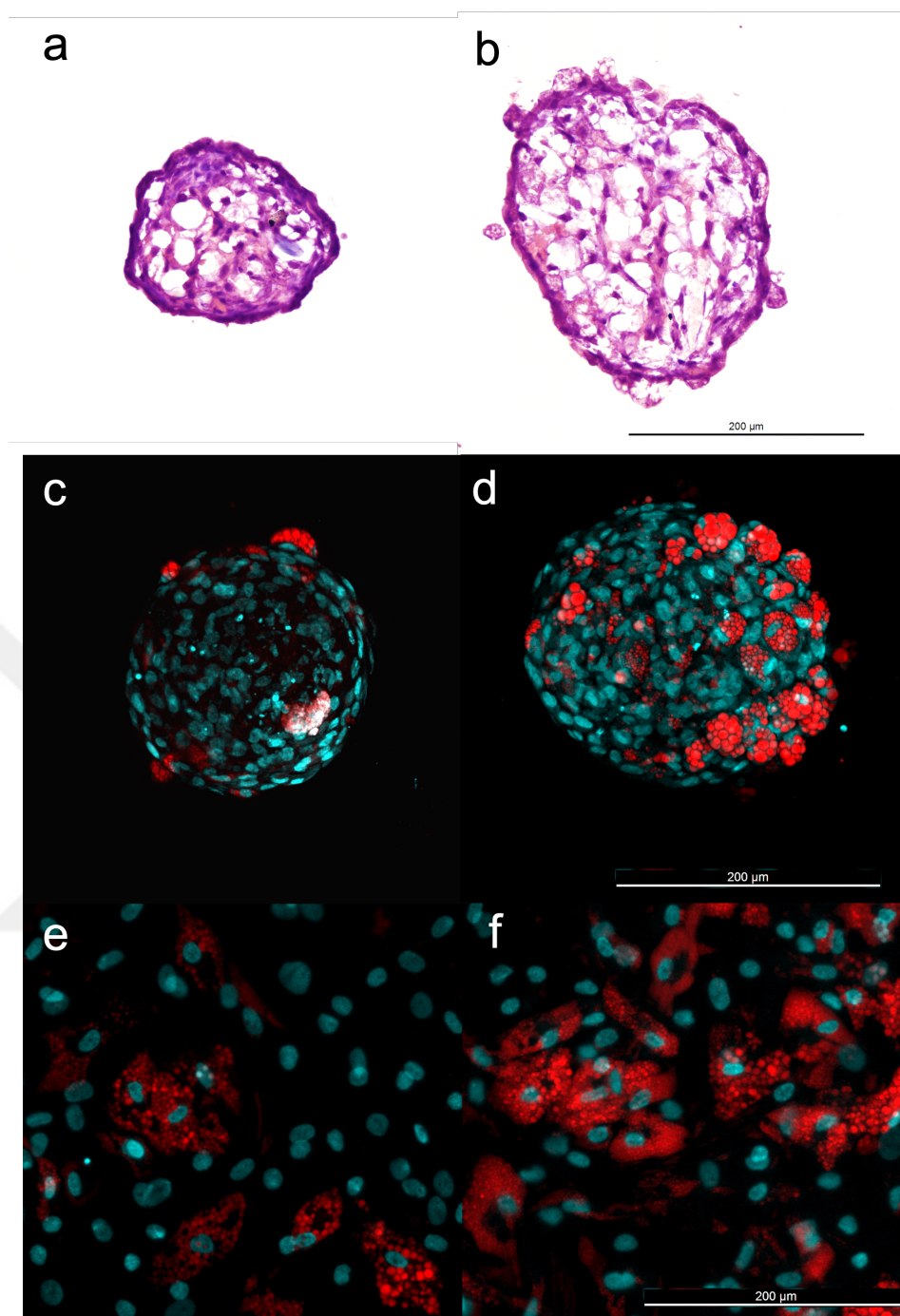
After optimization, the morphology of control (DMSO) and ROSI spheroids were compared by H&E staining and confocal imaging, including the examination of lipid droplet distribution and possible signs of necrosis in the inner core of the spheroid.

ROSI treatment increased the size of the spheroids in terms of lipid accumulation but did not affect Hoechst staining (Fig. 4.2), indicating no substantial increase in cell proliferation. H&E staining and confocal microscopy confirmed an even distribution of lipid droplets throughout the spheroid without any sign of cell death in the core (Fig. 4.3.a-d).

In 2D, fluorescence microscopy confirmed increased lipid accumulation with ROSI treatment (Fig. 4.3.e-f). When comparing 3D spheroids with 2D monolayer adipocytes, fewer and larger lipid droplets appear, compared to 2D, indicating a phenotype more similar to *in vivo* adipose tissue, especially visible with ROSI treatments.



**Figure 4.2** Effect of 0.5  $\mu$ M Rosiglitazone (ROSI) on cell proliferation (3D) as fold change (FC) to control (DMSO). Bars represent averages of 6 to 8 spheroids per condition, error bars indicate standard deviation (GraphPad Prism, v9.0).



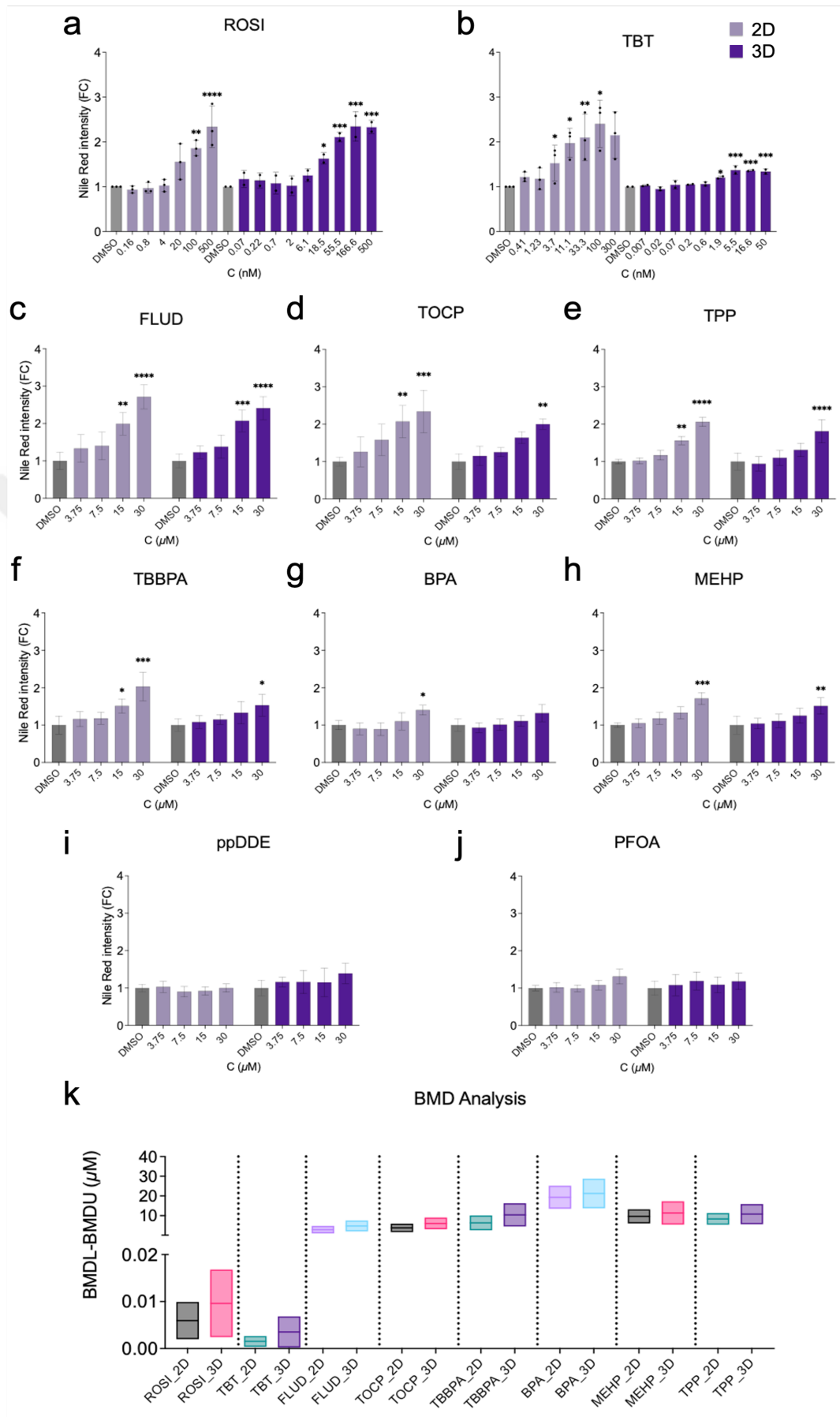
**Figure 4.3** Morphology of 3D spheroids. Brightfield microscopy images of 3D spheroids treated with (a) control (DMSO) and (b) positive control rosiglitazone (ROSI) ( $0.5 \mu\text{M}$ ). Staining: Hematoxylin & Eosin. Confocal microscopy images of 3D spheroids after (c) control and (d) positive control treatment. Staining: Nile Red (lipid droplets –red) and Hoechst (cell nuclei –cyan). Fluorescence microscopy images of 2D adipocytes after (e) control and (f) positive control treatment. Staining: Nile Red (lipid droplets –red) and Hoechst (cell nuclei –cyan). All images were taken under 20x magnification, scale bar: 200  $\mu\text{m}$ .



### ***Performance of the Models***

Following optimization, a battery of obesogens (see Chapter 2.2.1, Fig. 2.5) were tested in parallel experiments in 2D and 3D to assess the performance of the 3D model. The selection was based on known adipogenic activity of the chemicals and BMD modeling was employed to compare the adipogenic potencies of the different models. Obesogens exhibited similar effects on adipogenesis in both 2D and 3D models, confirmed by BMD modeling, which showed no significant difference in adipogenic potencies of the models. ROSI, TBT, BPA, TBBPA, FLUD, MEHP, TOCP, and TPP induced adipogenesis in both models, indicating the novel 3D model is suitable for assessing potential effects of chemicals on adipogenesis.

A similar induction was observed with ROSI on adipogenesis in both models (Fig. 4.4.a). While with TBT the results were more variable between the different models with a lower response in the 3D (Fig. 4.4.b). Both FLUD and TOCP induced adipogenesis in both models at 15 and 30  $\mu$ M, as potently as the positive control, ROSI (Fig. 4.4.c-d). Similarly, TPP and TBBPA exposure induced adipogenesis at 15 and 30  $\mu$ M, however, the effect was less compared to the other obesogens, and the induction was slightly higher in 2D (Fig. 4.4.e-f). BPA and MEHP only caused a slight induction at the highest tested dose of 30  $\mu$ M in both models (Fig. 4.4.g-h), whereas PFOA or *p,p'*-DDE exposure did not induce any effects in either model and were excluded from BMD modeling (Fig. 4.4.i-j). BMDs for these MDCs largely overlapped, indicating the comparability in adipogenic potencies of the two models (Fig. 4.4.k).



**Figure 4.4** Results of the battery of obesogens. Increase in neutral lipids during differentiation with (a) Rosiglitazone (ROSI); (b) Tributyltin (TBT); (c) Fludioxonil (FLUD); (d) Tri-ortho-cresyl phosphate (TOCP); (e) Triphenyl phosphate (TPP); (f) Tetrabromobisphenol A (TTBPA); (g) Bisphenol A (BPA); (h) Mono(2-ethylhexyl) phthalate (MEHP); (i) Dichlorodiphenyl-dichloroethylene (ppDDE/ p,p'-DDE) and (j) Perfluorooctanoic acid (PFOA) as fold change (FC) compared to control (DMSO). Bars represent averages of 3 independent experiments and error bars indicate standard deviation. (k) Benchmark dose (BMD) modeling of the obesogens for the different models. Boxes indicate 90% confidence intervals (BMDL-BMDU), and the lines represent median BMDs. \*  $p < 0.05$ ; \*\*  $p < 0.005$  \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$  (GraphPad Prism (v9.0)).

### ***Transcriptional Profiling of the Models***

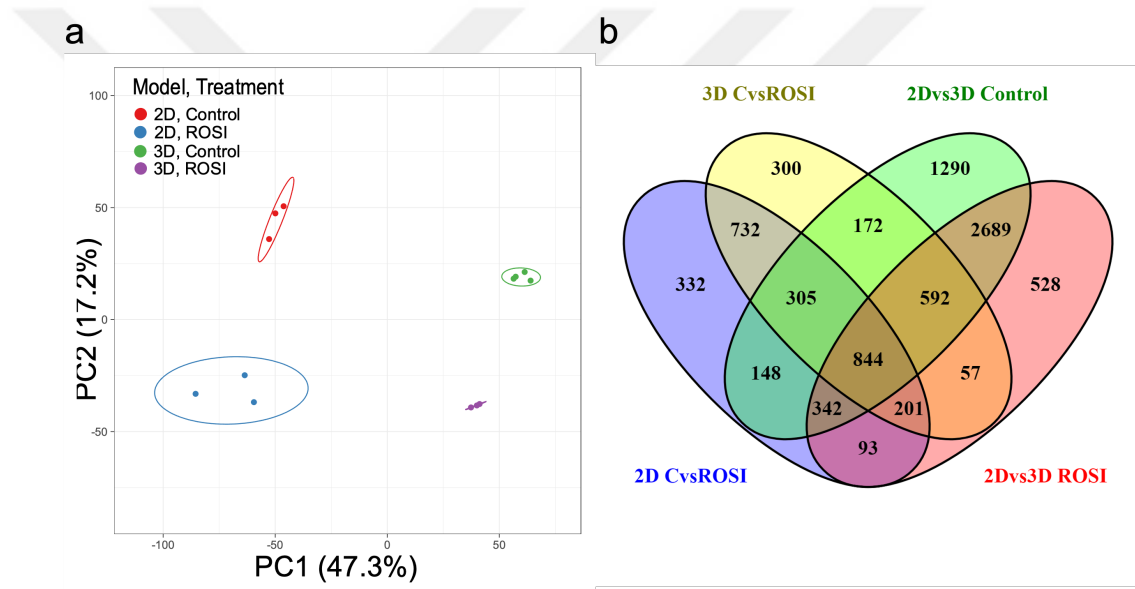
The 3D model was further characterized, by performing parallel RNA-seq analysis in the 2D and 3D model and comparing the transcriptional profiles of cells treated with either control (0.1 % DMSO) or positive control (0.1  $\mu$ M ROSI).

### ***Differential Gene Expression Analysis***

Differential gene expression analysis was performed, and the different treatments within the models (2D control vs. 2D ROSI and 3D control vs. 3D ROSI) were compared to assess the difference in potency and sensitivity to adipogenic pathways between the two models. Subsequently, to better assess the differences between the models regardless of the treatment, comparisons were made within the same treatment (2D control vs 3D control and 2D ROSI vs 3D ROSI).

Initial clustering of the data was done with a principal component analysis (PCA) plot based on the different models and treatments. PC1 axis shows the data is mostly clustered based on the model (47.3 %), with 2D data presented on the left and 3D data presented on the right side of the plot. PC2 axis shows additional clustering of the data based on the treatment (17.2 %). For both models, ROSI treatment showed separate clustering in the same direction compared to the control indicating a similar transcriptional profile (Figure 4.5.a).

Further exploration in DEGs revealed a 50 % overlap (2082) between the two models after differentiation with ROSI (Figure 4.5.b). 22 % (915) being exclusive for 2D ROSI, and 27 % (1121) exclusive for 3D ROSI. When looking at the two models within the same treatment the overlap ratio goes up to 61 % (4467), highlighting the similar expression profiles of the different models (Figure 4.5.b). 12 % (879) was differentially expressed only in the 3D model compared to the 2D during differentiation with ROSI, and 26 % (1915) was only differentially expressed during control treatment.



**Figure 4.5** Differential gene expression analysis. (a) Normalized reads per million of measured genes, presented in a PCA plot for the different treatment samples– 2D control (DMSO), 2D rosiglitazone (ROSI), 3D control, and 3D ROSI. Ellipses represent the 95% confidence intervals. (b) Venn diagram representation of overlapping differentially expressed genes (DEGs) across different treatments (FDR < 0.05).

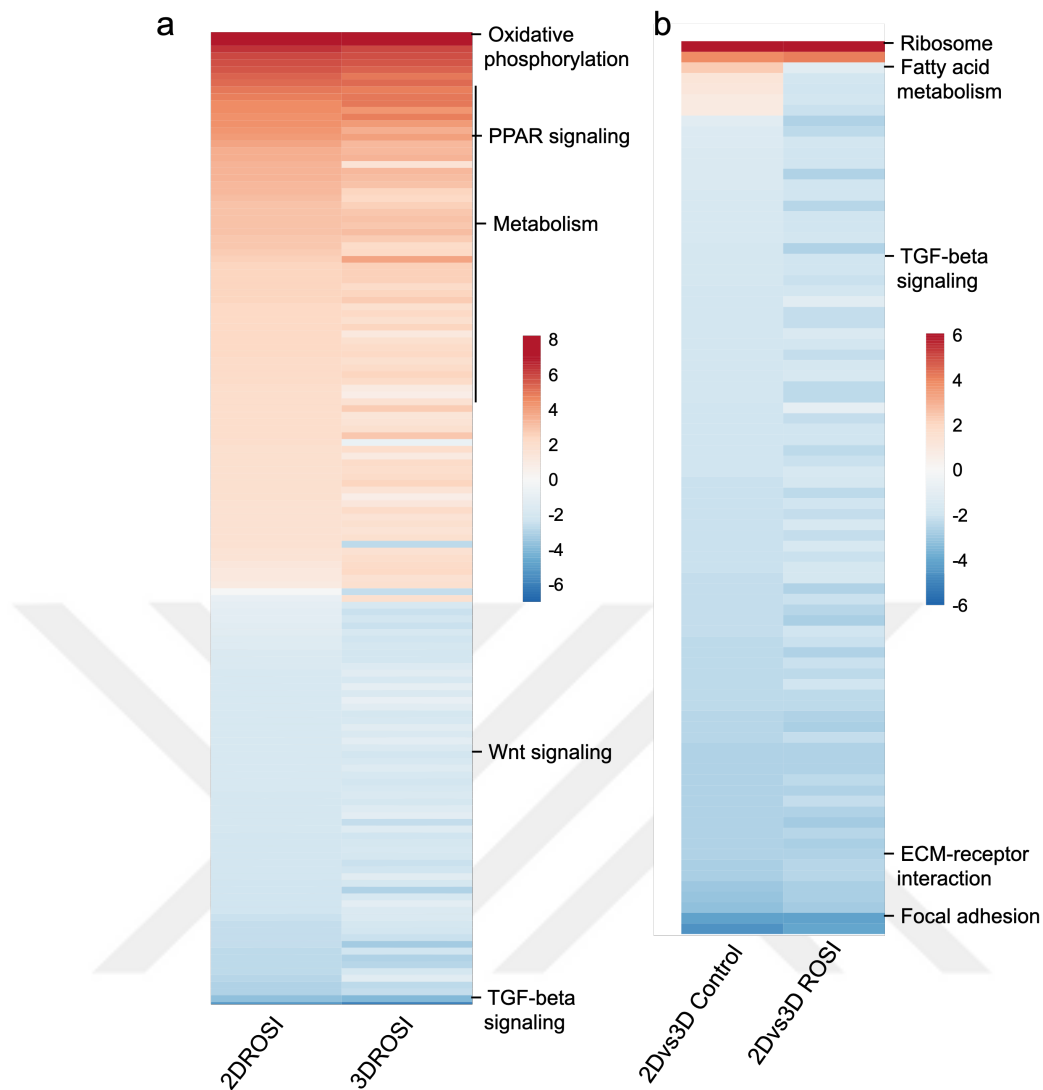
### ***Gene Set Enrichment and Pathway Analysis***

DEGs were scored based on significance and change in expression and used for gene set enrichment analysis (GSEA) via the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO Biological terms (GO Bio)) databases.

Pathways with a false discovery ratio below 5 % (FDR<0.05) were considered significant, revealing 75 upregulated and 54 downregulated KEGG pathways for the 2D ROSI treatment, as well as 65 upregulated and 80 downregulated GO Bio pathways. For the 3D ROSI treatment, the analysis revealed 70 upregulated and 44 downregulated KEGG pathways, along with 52 upregulated and 82 downregulated GO Bio pathways.

Most of the pathways were altered similarly during adipocyte differentiation with ROSI in both models. Adipogenic pathways essential for adipocyte differentiation, such as PPAR signaling, were upregulated; while pathways inhibiting adipogenesis, such as Wnt signaling and TGF- $\beta$  signaling, were downregulated. Notably, the oxidative phosphorylation pathway showed the strongest upregulation (Fig. 4.6.a).

The comparison of the different models within treatments revealed most pathways were downregulated in 3D compared to 2D, including metabolic pathways. Interestingly, the fatty acid metabolism pathway was upregulated in 3D compared to 2D, however only with the control treatment. Surprisingly, pathways related to cell interactions, such as extracellular matrix (ECM)-receptor interaction and focal adhesion, were found downregulated in 3D with both control and ROSI treatment, however many collagens were among the highest upregulated genes in the 3D model, indicating differences in structural integrity between both models (Fig. 4.6.b).



**Figure 4.6** Results of pathway analysis. Annotated heatmap of altered Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways highlighted based on significant down (blue) or upregulation (red) for at least one of the conditions (FDR < 0.05). (a) Rosiglitazone (ROSI) treatment compared to control conditions in 2D (left) and 3D (right), (b) within control (left) and ROSI (right) treatments in the same model. Pathways are ordered based on Normalized Enrichment Scores (NES), with row centering and row scaling disabled to visualize true NES scores.

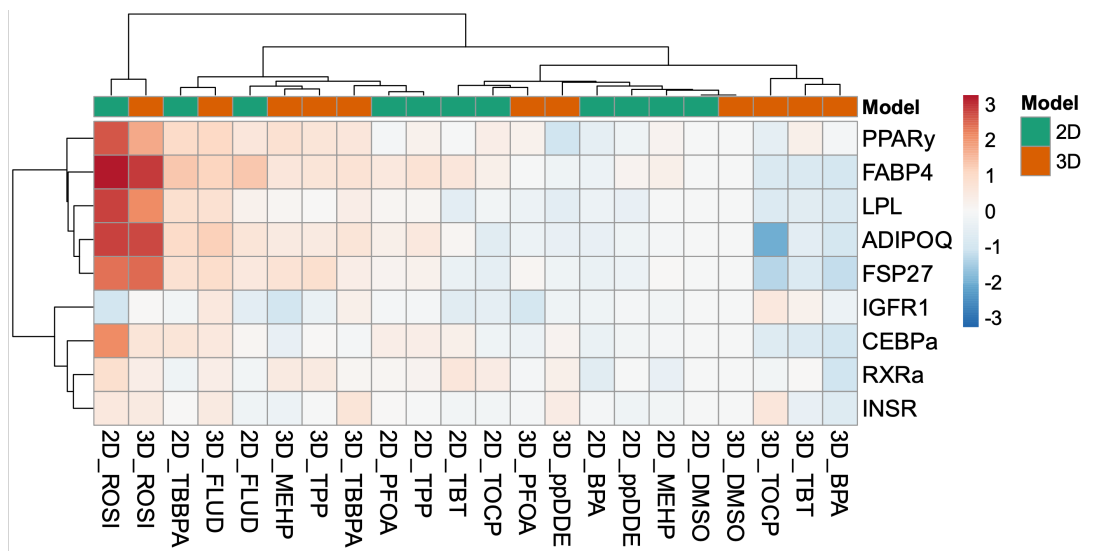
### *Assessment of Adipogenic Gene Expression*

To further clarify the underlying gene expression profiles between the two models, the expressions of selected adipocyte genes were investigated via QPCR analysis, following exposure to a broader class of obesogens (see previous chapter on assay performance). Selection of genes was based on their different roles in the process of adipogenesis, including regulators of adipogenesis (*PPAR $\gamma$* , *CEBPA*, and *RXR $\alpha$* ), *PPAR $\gamma$*  targets (*LPL*, *FABP4*, *ADIPOQ*, and *FSP27*), and genes involved in glucose homeostasis via insulin signaling (*INSR* and *IGF1R*).

ROSI was tested at 0.1  $\mu$ M, and TBT at 0.01  $\mu$ M, while the obesogens were tested at 15  $\mu$ M in parallel 2D 3D experiments.

Despite showing similar effects on lipid accumulation, gene expression revealed distinct transcriptomic profiles with the obesogens. Notably, exposures in the 2D model revealed mainly upregulation, while the same treatment in 3D led to downregulation (Fig. 4.7). Treatment with some of the obesogens revealed a reversed gene expression profile under 2D and 3D conditions. Clustering of TOCP, BPA, and TBT clearly shows the difference in expression profiles, with 3D treatments, presented on the right side of the heatmap, leading to stronger downregulation compared to 2D. Remarkably, TOCP treatment in 3D caused the strongest downregulation of *ADIPOQ*, and upregulation of *INSR* and *IGF1R*, genes involved in insulin and adipocytokine signaling. These effects were not apparent in 2D, indicating mechanisms of actions other than *PPAR $\gamma$*  activation might be undetected in the 2D model, but become evident under more complex 3D conditions.

Conversely, ROSI and FLUD treatments can be seen clustering together on the left side of the heatmap, leading to upregulation of *PPAR $\gamma$*  and its' targets with comparable expression profiles in both models. Notably, with ROSI treatment there is a stronger upregulation in the 2D model compared to 3D, while FLUD treatment led to a stronger upregulation in 3D than 2D.

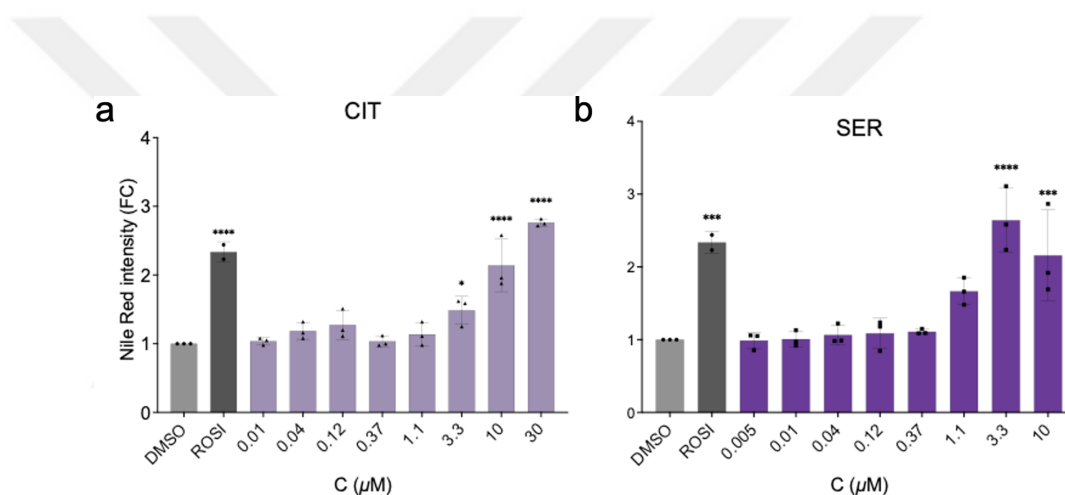


**Figure 4.7** Heatmap representation of adipocyte gene expression via QPCR analysis. Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), CAAT/enhancer-binding protein alpha (CEBP $\alpha$ ), retinoid X receptor alpha (RXR $\alpha$ ), lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4), adiponectin (ADIPOQ), fat-specific protein 27 (FSP27), insulin receptor (INSR), and insulin-like growth factor 1 receptor (IGF1R). Color scale based on log<sub>2</sub> normalized expression values calculated via the  $\Delta\Delta C_q$  method (red– higher, blue– lower expression). Chemicals were tested at 15  $\mu$ M, Rosiglitazone (ROSI) was used as positive control at 0.1  $\mu$ M. Rows and columns clustered using Euclidean distance and Ward linkage (ClustVis).



#### 4.1.1.2. Assessment of the Adipogenic Potential of SSRIs

Starting first with the fully characterized 3D model, the adipogenic potential of CIT and SER was investigated. Where an increase in neutral lipids, an indicator of enhanced adipogenesis, was observed with both SSRIs (Fig. 4.8.a-b). The induction was comparable to positive control, ROSI at 0.5  $\mu$ M. BMD analysis revealed lower and upper thresholds (BMDL-BMDU) of 1.32-3.27  $\mu$ M for CIT, and 0.35-0.87  $\mu$ M for SER, for a 1.2-fold induction, confirming the SSRIs' effect on adipogenesis was observed close to their reported SSCs (0.12-0.92  $\mu$ M for CIT and 0.065-0.65  $\mu$ M for SER) (Baumann 1996; De Vane et al. 2002).



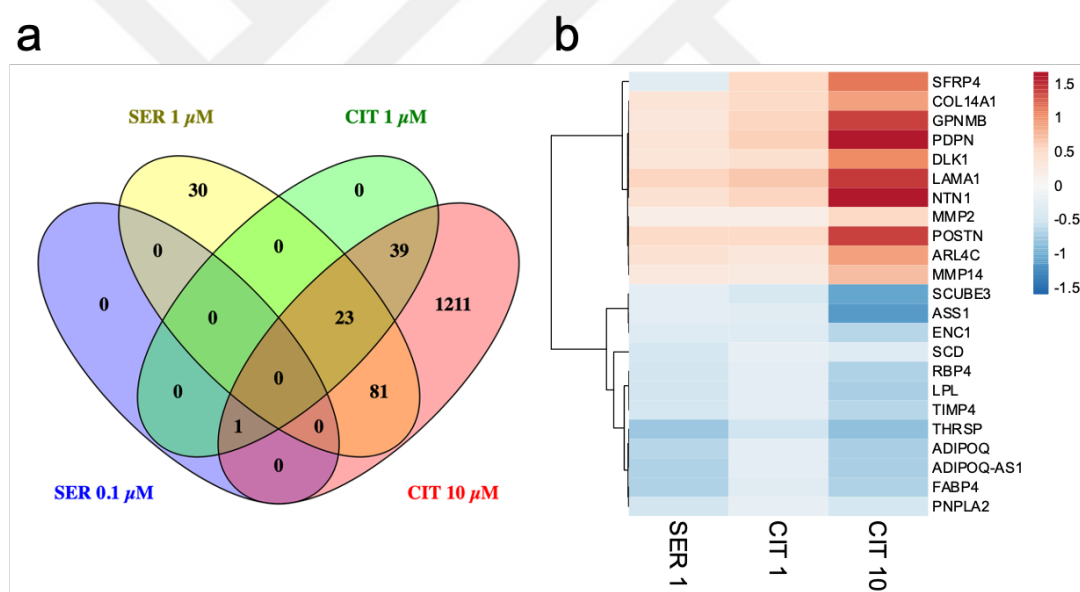
**Figure 4.8** Effects on 3D spheroids. The increase in neutral lipids during differentiation with (a) Citalopram (CIT), and (b) Sertraline (SER) as fold change (FC) compared to control (DMSO). ROSI: Rosiglitazone (0.5  $\mu$ M). Bars represent averages of 3 independent experiments and error bars indicate standard deviation. \*\*\* p < 0.0005; \*\*\*\* p < 0.0001 (GraphPad Prism, v9.0).

#### *SSRIs' Effects on the Transcriptome*

To clarify the mechanisms of action of the SSRIs, transcriptional profiling was performed on cells treated with control (0.1 % DMSO), positive control (0.1  $\mu$ M ROSI) or CIT and SER in the 3D model by RNA-seq. CIT was tested at 1  $\mu$ M (NOEL) and 10  $\mu$ M (LOEL), SER was tested at 0.1  $\mu$ M (NOEL) and 1  $\mu$ M (LOEL).

### Differential Gene Expression Analysis

For the SSRIs, differential gene expression analysis revealed a single DEG for SER 0.1  $\mu\text{M}$  “*insulin like growth factor-2*”, which was downregulated, and 134 genes for SER 1  $\mu\text{M}$ , of which 63 % (85) were downregulated. CIT treatments mainly resulted in upregulation, 71 % (45) of 63 genes were upregulated for CIT 1  $\mu\text{M}$ , and 57 % (783) of 1355 genes were upregulated for CIT 10  $\mu\text{M}$ . A total of 23 genes were found overlapping between SER 1  $\mu\text{M}$  and CIT treatments (Fig. 4.9.a). Among these, there were some associated with adipogenesis or lipid metabolism including adiponectin, lipoprotein lipase, and fatty acid binding protein 4 (*ADIPOQ*, *LPL*, *FABP4* respectively). Surprisingly, these were downregulated with all the treatments (Fig. 4.9.b).



**Figure 4.9** Differential gene expression analysis. (a) Venn diagram representation of overlapping differentially expressed genes (DEGs) (FDR < 0.05) for SER (0.1 and 1  $\mu\text{M}$ ) and CIT (1 and 10  $\mu\text{M}$ ). (b) Heatmap with normalized counts of 23 overlapping DEGs among 1  $\mu\text{M}$  SER and CIT (1 and 10  $\mu\text{M}$ ) treatments.

### ***Gene Set Enrichment and Pathway Analysis***

Following ranking of genes based on their significance and fold change (see Methods), GSEA analysis revealed a generally reversed gene expression profile compared to the positive control, ROSI (Fig. 4.10.a). Metabolic pathways such as PPAR signaling, non-alcoholic fatty liver disease (NAFLD) and lipid metabolism were found consistently downregulated, in a concentration dependent manner, with the SSRI treatments (Fig. 4.10.a), in the functional network of SER and CIT these pathways can be seen clustering together (Fig. 4.10.b). Notably, the lysosome pathway was upregulated by all treatments (Fig. 4.10.c), and this effect was significant for SER 1  $\mu$ M and both CIT concentrations (FDR<0.05) (Fig. 4.10.c).

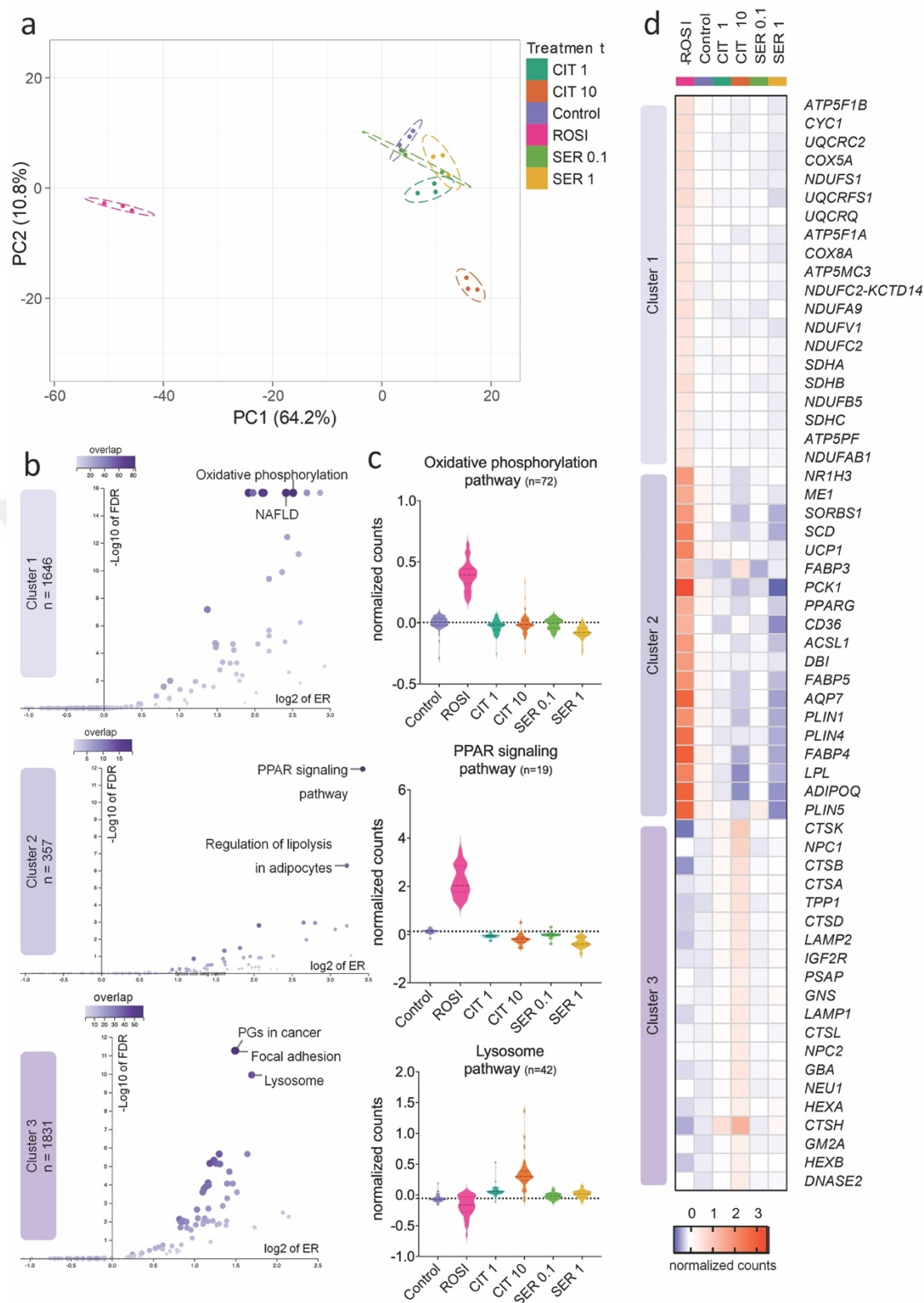




### ***K-means Clustering and Pathway Analysis***

Further analyses on the RNA-seq data was performed for an in-depth assessment of the differences in gene expression profiles between ROSI and the SSRIs. Fig. 4.11.a shows the distribution of all genes for each replicate sample, clearly showing the separation between ROSI and SSRI treatments. 3848 genes were significantly affected by at least one of the treatments ( $FDR < 0.05$ ) and separated into 3 clusters using k-means clustering. Quality of the clustering was confirmed by silhouette scoring, and an over representation analysis (ORA) was performed by using the genes within the clusters, and focusing on KEGG pathways. First two clusters both contain metabolic pathways that are upregulated by ROSI and downregulated consistently by SSRI treatments. First cluster consists of 1646 DEGs, leading to 128 enriched pathways. Among these are oxidative phosphorylation and NAFLD pathways, with higher enrichment ratios and lowest FDR values compared to other pathways (Fig. 4.11.b). The second cluster is representing adipocyte differentiation with pathways such as PPAR signaling, regulation of lipolysis in adipocytes and other metabolic pathways (Fig. 4.11.b). It consists of 172 mapped genes and 66 enriched pathways in total. The final cluster has the highest number of enriched pathways, with 196 pathways from 750 mapped genes, and contains genes consistently upregulated by SSRIs and downregulated by ROSI, among which the lysosome pathway as one of the most significant pathways (Fig. 4.11.b).

In Fig. 4.11.c violin plots of the representative pathways from each cluster are shown. The upregulation of PPAR signaling pathway following ROSI treatment is much stronger, as expected. Among the top transcripts upregulated by ROSI are many well-characterized gene targets of *PPAR* $\gamma$ , including *FABP4*, *ADIPOQ*, *LPL*, *PLIN4* (*perilipin 4*), *PLIN5* (*perilipin 5*), and *PCK1* (*phosphoenol-pyruvate carboxykinase 1*) (Fig. 4.11.c). Whereas these are downregulated by the SSRI treatments, in a concentration dependent manner (Fig. 4.11.d). For the lysosome pathway cathepsins (*CTS*), a family of lysosomal proteases, are among the highest upregulated genes with the SSRIs treatments, especially with CIT at 10  $\mu$ M, while being downregulated by ROSI (Fig. 4.11.d).



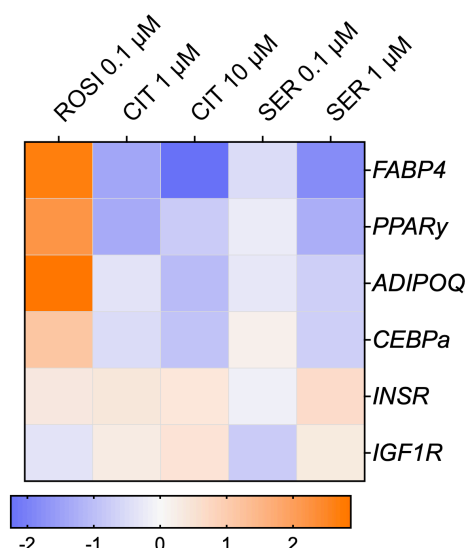
**Figure 4.11** K-means clustering and pathway analysis. (a) Normalized counts of all genes for each replicate sample (Control (DMSO), Rosiglitazone (ROSI), Citalopram (CIT 1–10  $\mu$ M), and Sertraline (SER 0.1–1  $\mu$ M)), presented as principal component analysis (PCA) plot (ClustVis). Ellipses represent the 95 % confidence intervals. (b)

Annotated pathways with higher enrichment ratio (ER) and significance (n=number of genes per cluster). Scale bar shows the number of overlapping genes between mapped input and gene set. NAFLD: Non-alcoholic fatty liver disease; PPAR: Peroxisome proliferator-activated receptor; PGs: Proteoglycans; FDR: False Discovery Rate. (c) Plots of median-adjusted normalized counts of the genes within representative pathways (n=overlap between mapped input and gene set), dashed lines set at median values of control treatment. (d) Heatmap with median-adjusted normalized counts of 20 representative genes from each pathway (GraphPad Prism, v9.0).

### ***SSRIs' Effects on Adipogenic Gene Expression***

To confirm the findings of RNA-seq analysis, QPCR analysis on selected adipocyte genes was performed (see previous chapter on adipogenic gene expression with the battery of obesogens).

A similar expression profile was observed with the SSRIs, confirming the results of the RNA-seq. Genes from the PPAR signaling pathway including *PPAR $\gamma$* , *CEBP $\alpha$* , *FABP4*, and *ADIPOQ* were found downregulated after SSRI treatments, contrary to ROSI, where a strong induction was observed (Fig. 4.15). Notably, genes involved in insulin signaling, *INSR* and *IGF1R*, were slightly upregulated by the SSRIs, with the exception of SER at the lowest tested concentration (0.1  $\mu$ M) (Fig. 4.12).



**Figure 4.12** Expression of selected adipocyte genes in 3D spheroids after Rosiglitazone (ROSI 0.1  $\mu$ M), Citalopram (CIT 1–10  $\mu$ M) and Sertraline (SER 0.1–1  $\mu$ M) treatments. Color scale based on log2 normalized expression values (orange representing higher and blue representing lower expression) (GraphPad Prism, v9.0). FABP4: Fatty acid binding protein 4, PPAR $\gamma$ : Peroxisome proliferator-activated receptor gamma, ADIPOQ: Adiponectin, CEBP $\alpha$ : CAAT/enhancer-binding protein alpha, INSR Insulin receptor, IGF1R: Insulin-like growth factor 1 receptor.

### ***SSRIs' Effects on Phospholipids and Lysosomes***

The unexpected results of the RNA-seq were followed-up by further experiments with functional readouts in the 2D model for a complete understanding of SSRIs' effects on adipogenesis.

#### ***During MSC Differentiation***

Starting with the SSRIs' effects on different lipid profiles during and after adipocyte differentiation, the 2D model was employed followed by flow cytometry analysis. Both neutral and phospholipids were quantified by measuring Nile Red intensity in two different channels (see methods). Fluorescence microscopy image of Nile Red in the green- and red-fluorescent channels (Fig. 4.13.a) shows the separation between lipids, proving the method's applicability in quantifying both neutral and phospholipids.

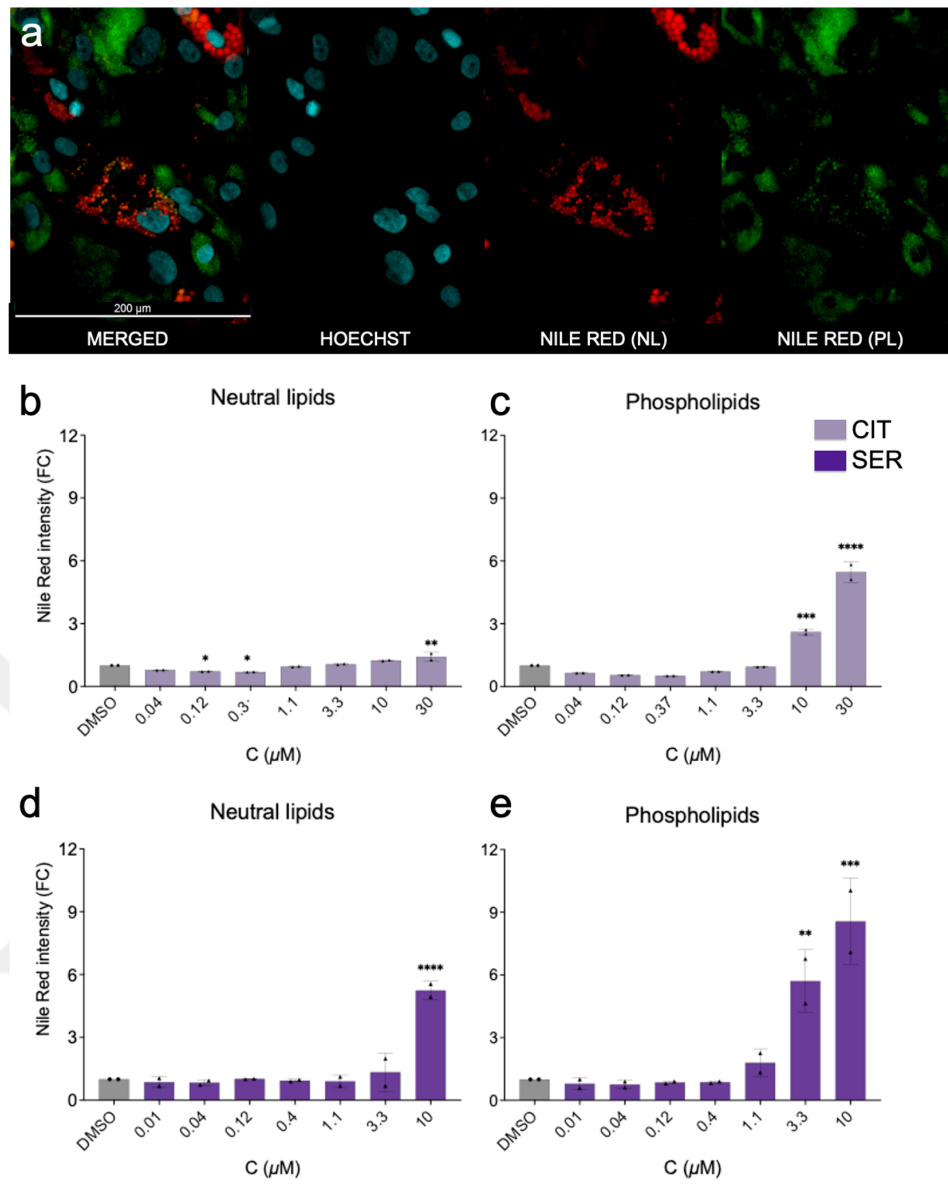


As expected, CIT and SER both increased neutral lipids in 2D MSCs during differentiation (Fig. 4.13.b-d). However, a much stronger induction was observed in the phospholipid channel with both SSRIs (Fig. 4.13.c-e). BMD modeling confirmed that SSRIs had a stronger effect on phospholipids. By comparing BMDs, we observed that SER induced phospholipids at nearly 10 times lower concentrations than neutral lipids (Table 4.1).

**Table 4.1** Benchmark dose (BMD) for a 20 % increase in neutral (NL) and phospholipids (PL) with SSRIs, including 90 % confidence intervals (BMDL-BMDU) and median BMDs (BMD<sub>MED</sub>).

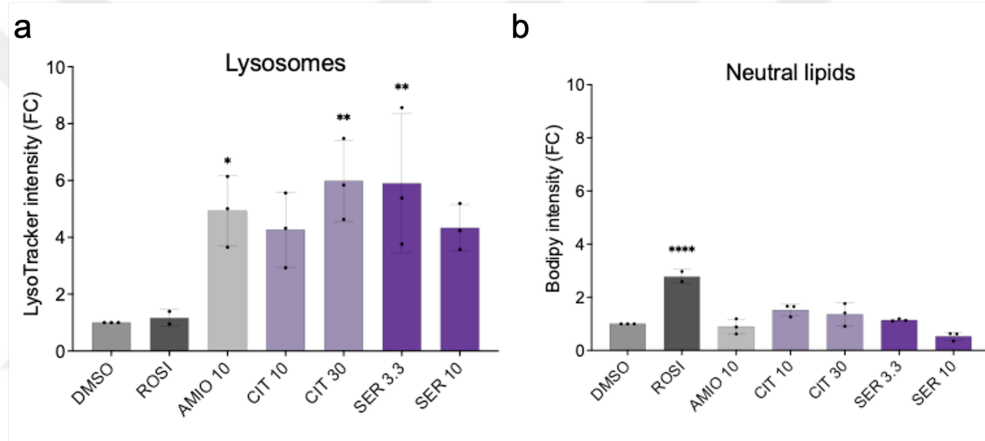
|            |    | <i>BMDL</i> ( $\mu$ M) | <i>BMDU</i> ( $\mu$ M) | <i>BMD<sub>MED</sub></i> ( $\mu$ M) |
|------------|----|------------------------|------------------------|-------------------------------------|
| <i>SER</i> | NL | 1.89                   | 6.35                   | 4.12                                |
|            | PL | 0.26                   | 0.66                   | 0.46                                |
| <i>CIT</i> | NL | 1.12                   | 4.80                   | 2.96                                |
|            | PL | 1.39                   | 3.15                   | 2.27                                |

Furthermore, the effect on phospholipids with SER was observed in a range around reported SSCs (0.065–0.65  $\mu$ M) (De Vane et al. 2002). Although CIT showed a strong induction in the phospholipid channel, BMD analyses revealed a similar range compared to neutral lipids and above reported SSCs. Notably, the confidence interval of the BMD of phospholipids was lower compared to neutral lipids, indicating a more accurate estimate of the BMD.



**Figure 4.13** (a) Fluorescence microscopy image of 2D adipocytes stained with Nile Red and Hoechst (merged). Split channels showing cell nuclei (DAPI channel– cyan), neutral lipids (NL) (FITC channel–red), and phospholipids (PL) (Texas Red channel–green), respectively. Scale bar: 200  $\mu$ m. The increase in (b) neutral– and (c) phospholipids during differentiation with Citalopram (CIT). The increase in (d) neutral– and (e) phospholipids during differentiation with Sertraline (SER). Results presented as fold change (FC) compared to control (DMSO). Bars represent averages of 2 independent experiments and error bars indicate standard deviation. \*  $p < 0.05$ ; \*\*  $p < 0.005$  \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$  (GraphPad Prism, v9.0).

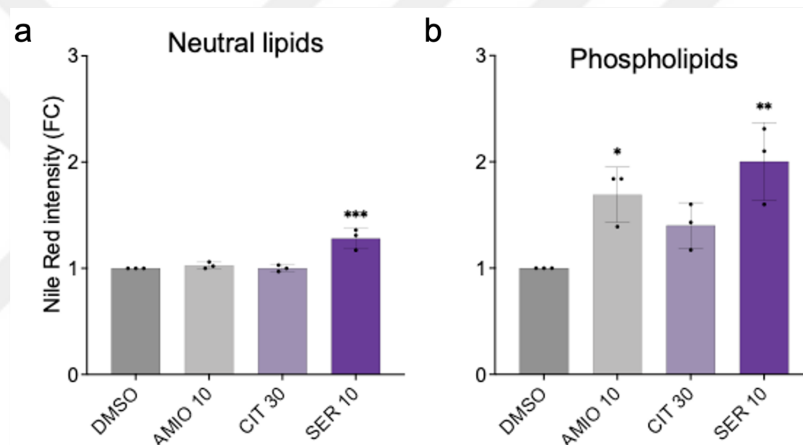
Next, the SSRIs' effects on lysosomes during differentiation was studied via LysoTracker Red staining. Amiodarone (AMIO) at 10  $\mu$ M was used as positive control, while CIT and SER were tested at 10–30  $\mu$ M and 3.3–10  $\mu$ M, respectively. Here, a strong inducing effect on lysosomes was observed with both CIT and SER (Fig. 4.14.a). SSRIs at the tested concentrations led to an induction as high as the positive control. Neutral lipids were assessed by Bodipy staining, where the induction was minimal, moreover, a slight decrease was observed with SER at 10  $\mu$ M (Fig. 4.14.b).



**Figure 4.14** Increase in (a) lysosomes and (b) neutral lipids in 2D MSCs during differentiation with citalopram (CIT 10–30  $\mu$ M), and sertraline (SER 3.3–10  $\mu$ M). Presented as fold change (FC) compared to control (DMSO). Bars represent averages of 3 independent experiments and error bars indicate standard deviation. ROSI: Rosiglitazone (0.5  $\mu$ M), AMIO: Amiodarone (10  $\mu$ M). \*  $p < 0.05$ ; \*\*  $p < 0.005$  \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$  (GraphPad Prism, v9.0).

### *Mature Adipocytes*

As the patients receiving SSRI treatment are mostly adults, additional experiments were performed on mature adipocytes to study the effects on MSCs after differentiation to see if SSRIs would exert the same effect, changing the lipid profile from neutral lipids to phospholipids. A slight increase in neutral lipids was observed with only SER at 10  $\mu$ M after differentiation (Fig. 4.15.a). However, phospholipid levels increased, suggesting SSRIs can also increase phospholipid accumulation in mature adipocytes (Fig. 4.15.b).

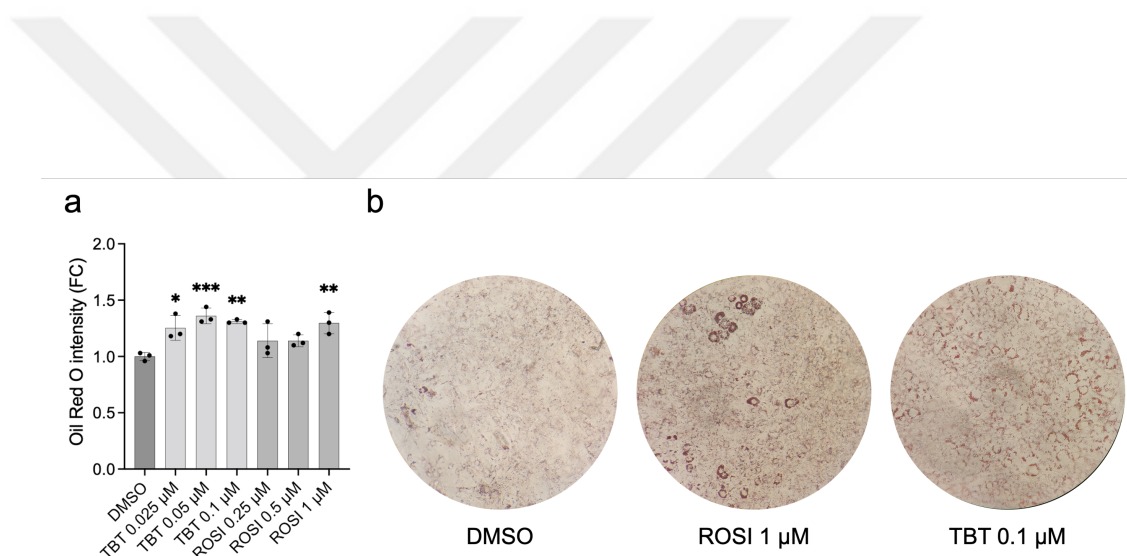


**Figure 4.15** Effects on mature adipocytes. Increase in (a) neutral and (b) phospholipids by citalopram (CIT 30  $\mu$ M), sertraline (SER 10  $\mu$ M), and amiodarone (AMIO 10  $\mu$ M), presented as fold change (FC) compared to control. Bars represent averages of 3 independent experiments and error bars indicate standard deviation. \*  $p < 0.05$ ; \*\*  $p < 0.005$  \*\*\*  $p < 0.0005$  (GraphPad Prism, v9.0).

#### 4.1.2. 3T3-L1 Preadipocyte Model

To study the effects of SSRIs using the 3T3-L1 cell line, the performance of the model was first determined by using reference compounds, prototypical *PPAR* $\gamma$  ligand, ROSI and the well-studied obesogen TBT. As part of partial validation studies, ROSI and TBT were tested at 3 concentrations (ROSI: 0.25, 0.5, 1  $\mu$ M, TBT: 0.025, 0.05, 0.1  $\mu$ M).

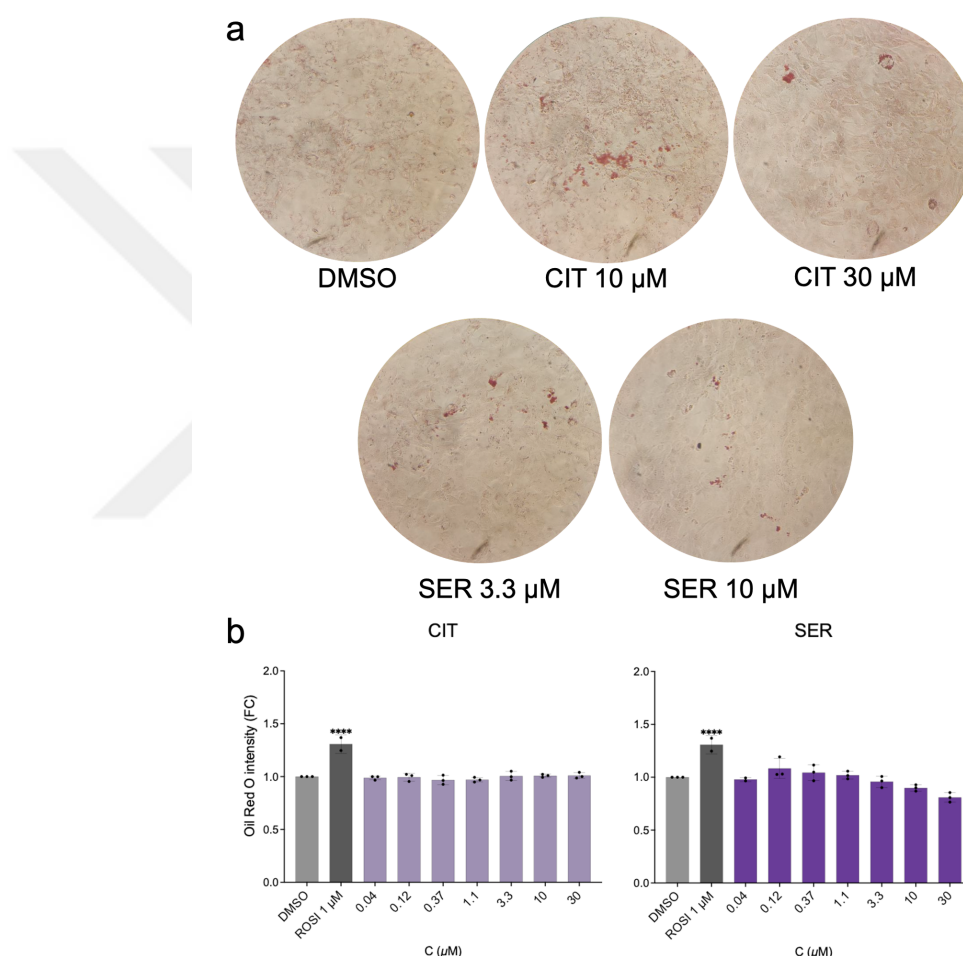
A significant increase in lipid accumulation was observed with TBT, and at the highest tested concentration of ROSI (1  $\mu$ M), compared to control (DMSO) (Fig. 4.16.a). Brightfield microscopy confirmed the increase in lipid accumulation and the number of adipocytes with both ROSI (1  $\mu$ M) and TBT (0.1  $\mu$ M) (Fig. 4.16.b).



**Figure 4.16** (a) Increase in neutral lipids measured with rosiglitazone (ROSI) and tributyltin (TBT), as fold change (FC) compared to control (DMSO). Bars represent averages of 3 independent experiments and error bars indicate standard deviation. \* p < 0.05; \*\* p < 0.005 \*\*\* p < 0.0005 (GraphPad Prism (v9.0)). (b) Brightfield microscopy images of Oil Red O-stained adipocytes following differentiation with reference compounds (4x magnification).

#### 4.1.2.1. Assessment of the Adipogenic Potential of SSRIs

SSRIs' effects on 3T3-L1 differentiation was evaluated via brightfield microscopy (Fig. 4.17.a) and Oil Red O staining. An increase in neutral lipids was not observed with CIT at the tested concentrations (Fig. 4.17.b). Notably, a slight decrease was observed with SER that seemed to be concentration dependent, yet these effects were found non-significant compared to vehicle control (Fig. 4.17.b).



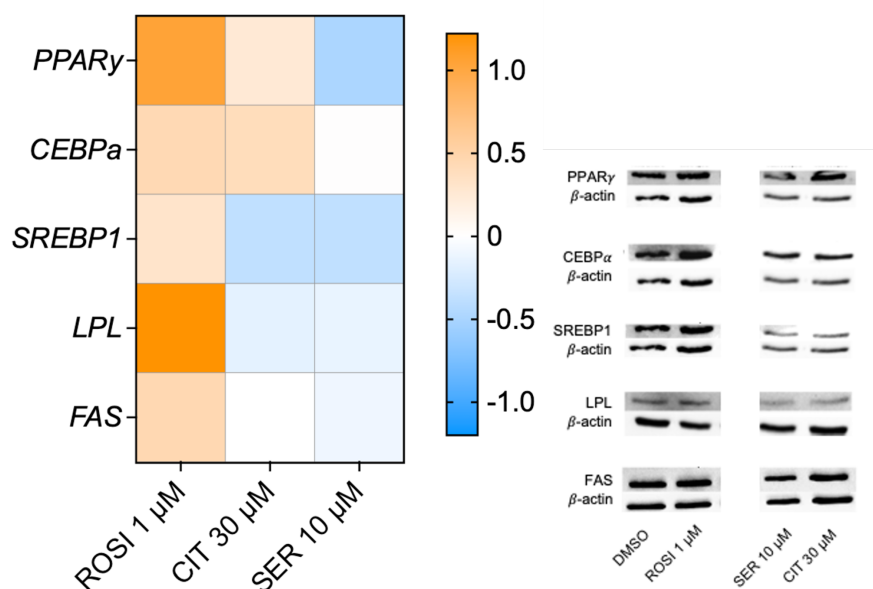
**Figure 4.17** (a) Brightfield microscopy images of Oil Red O-stained adipocytes following differentiation with citalopram (CIT) and sertraline (SER) (4x magnification). (b) Changes in neutral lipids with CIT and SER, as fold change (FC) compared to control (DMSO). Bars represent averages of 3 independent experiments and error bars indicate standard deviation. ROSI: Rosiglitazone. \*\*\*\*  $p < 0.0001$  (GraphPad Prism (v9.0)).

### ***SSRIs' Effects on Adipogenic Protein Expression***

To further investigate the effects of SSRIs on 3T3-L1 cells during differentiation, expression of selected adipogenic proteins (PPAR $\gamma$ , C/EBP $\alpha$ , SREBP1, LPL, and FAS) was evaluated with western blot analysis. ROSI at 1  $\mu$ M was used as positive control, while CIT and SER were tested at 30 and 10  $\mu$ M, respectively. Protein expression was normalized to the reference protein band (beta-actin) and the change in expression was calculated as log2 fold change over control (0.1 % DMSO).

ROSI treatment increased adipogenic protein expression up to different extent (Fig. 4.18). As expected, a strong induction was observed for PPAR $\gamma$ , as well as its downstream target, LPL. CEBP $\alpha$ , SREBP1, and FAS were also induced, although to a lesser extent.

CIT was found to slightly induce PPAR $\gamma$  and CEBP $\alpha$  levels (Fig. 4.18). Conversely, SER led to an overall decrease in adipogenic protein expression (Fig. 4.18), these changes align with the decrease in lipid accumulation observed with SER (Fig.4.17.b). Overall, altered expression of key adipogenic proteins suggests potential impact on adipogenesis by the SSRIs.



**Figure 4.18** Effects of citalopram (CIT) and sertraline (SER) on adipogenic protein expression. Color scale based on log2 normalized expression values compared to control (DMSO) (GraphPad Prism (v9.0)). PPAR $\gamma$ : Peroxisome proliferator-activated receptor gamma, CEBP $\alpha$ : CAAT/enhancer-binding protein alpha, SREBP1: Sterol regulatory element-binding protein 1, LPL: Lipoprotein lipase, FAS: Fatty acid synthase.

## 4.2. Assessment of Other Possible Mechanisms of the SSRIs

To investigate whether there are other mechanisms underlying SSRIs' effects, reporter cell lines were used for various nuclear receptors. Nuclear receptor-mediated activities were investigated for the PPARs (PPAR $\gamma$  and PPAR $\alpha$ ), ER, AR, and DR via receptor binding and transactivation assays. To further evaluate estrogenic effects, ER-dependent cell proliferation and cell-free aromatase inhibition assays were performed.

### 4.2.1. Receptor Binding and Transactivation Assays

A total of 10 endpoints were assessed by the reporter cell assays, including agonist and antagonist activity. A summary showing the endpoints and SSRIs' activities can be seen in Table 4.2. PPAR-mediated activity was not observed with CIT or SER at the



tested concentrations, indicating a different mechanism of action than binding to PPAR behind enhanced adipogenesis with SSRIs. Similarly, agonist or antagonist activity was not observed for either ER, AR or DR with the SSRIs at the tested concentrations, indicating a different mechanism of action than ER, AR, or DR activation behind their adipogenic effects.

**Table 4.2** Summary of the reporter cell assays.

|                                | <i>CIT-Ago</i> | <i>CIT-Anta</i> | <i>SER-Ago</i> | <i>SER-Anta</i> |
|--------------------------------|----------------|-----------------|----------------|-----------------|
| <i>PPAR<math>\gamma</math></i> | -              | -               | -              | -               |
| <i>PPAR<math>\alpha</math></i> | -              | -               | -              | -               |
| <i>ER</i>                      | -              | -               | -              | -               |
| <i>AR</i>                      | -              | -               | -              | -               |
| <i>DR</i>                      | -              | -               | -              | -               |

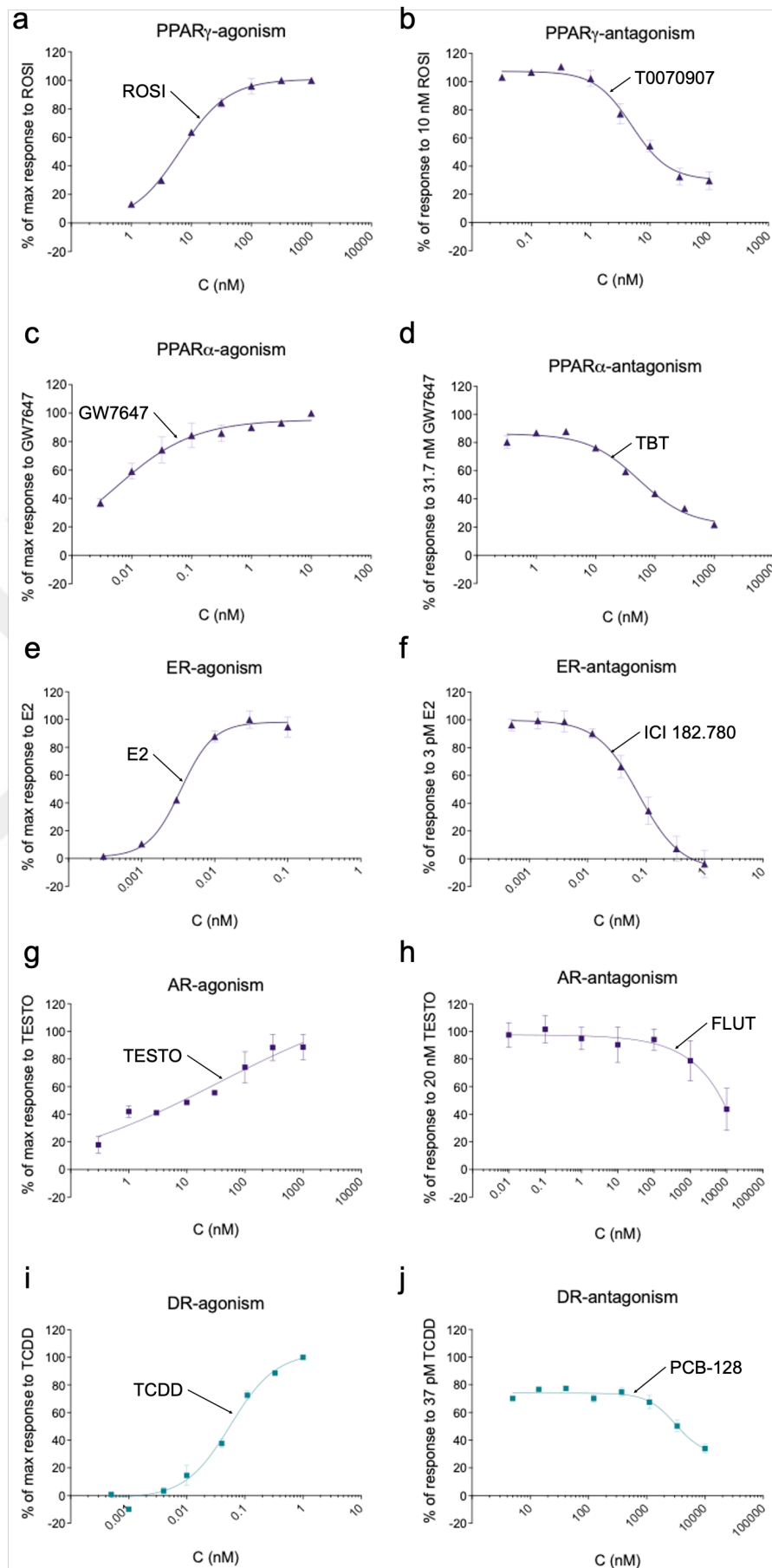
Concentration-response curves were generated with reference chemicals in the agonism and antagonism assays (Fig. 4.19.a-j). Half maximal effective concentrations (EC<sub>50</sub>) determined in the agonism assays were used to activate the receptor in the antagonism assays (Table 4.3).

Cell viability was determined via mitochondrial activity (Alamar Blue assay). SER at 30  $\mu$ M was found to be cytotoxic for both ER-Luc and AR-Luc cells (data not shown), therefore used at the highest concentration of 10  $\mu$ M for the following experiments. CIT did not decrease cell viability at the highest tested concentration of 30  $\mu$ M.

**Table 4.3** Reference compounds used in the reporter cell assays.

|                        | <i>PPAR<math>\gamma</math>-Luc</i> | <i>PPAR<math>\alpha</math>-Luc</i> | <i>ER-Luc</i>      | <i>AR-Luc</i> | <i>DR-GFP</i> |
|------------------------|------------------------------------|------------------------------------|--------------------|---------------|---------------|
| <i>Agonist</i>         | ROSI                               | GW7647                             | $\beta$ -Estradiol | Testosterone  | TCDD          |
| <i>EC<sub>50</sub></i> | 10 nM                              | 31.7 nM                            | 3 pM               | 20 nM         | 37 pM         |
| <i>Antagonist</i>      | T0070907                           | TBT                                | ICI 182,780        | FLUT          | PCB-128       |

EC<sub>50</sub>: Half maximal effective concentrations.

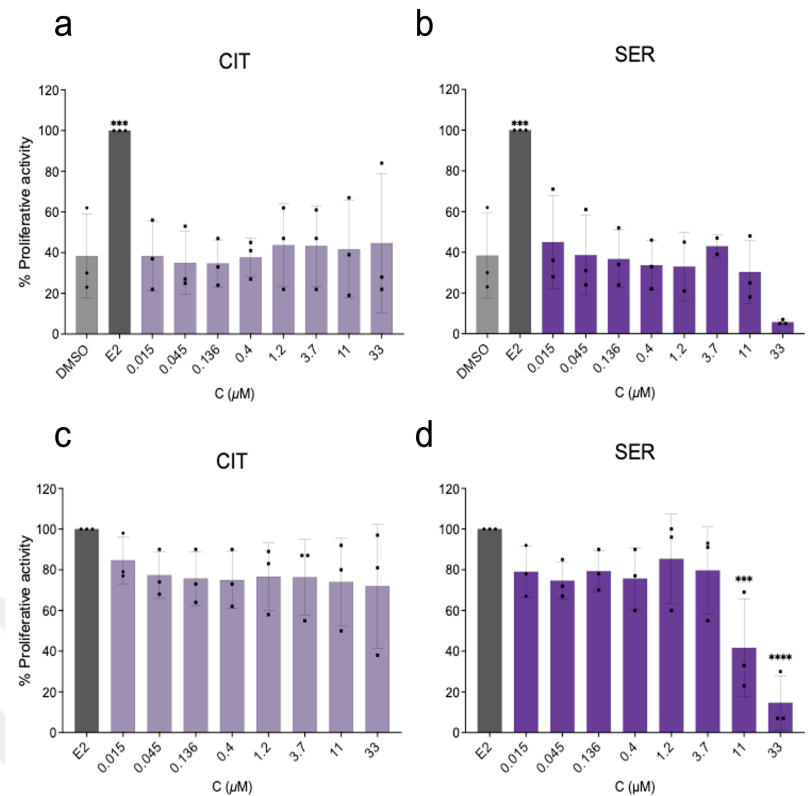


**Figure 4.19** (a-j) Concentration–response curves of reference compounds for the reporter cell assays. PPAR $\gamma$ , PPAR $\alpha$ : Peroxisome proliferator-activated receptors gamma and alpha, ROSI: Rosiglitazone, TBT: Tributyltin, ER: Estrogen receptor, E2:  $\beta$ -Estradiol, AR: Androgen receptor, TESTO: Testosterone, FLUT: Flutamide, DR: Dioxin receptor, TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin, PCB-128: 2,3,4,2',3',4'-Hexachlorobiphenyl. (GraphPad Prism, v9.0).

#### **4.2.2. ER-dependent Proliferation Assay**

To confirm the lack of ER-mediated activity by the SSRIs, the proliferation of the ER (+) MCF7 cell line was investigated. In the assay, chemicals with estrogenic activity activate the ER, subsequently enhancing MCF-7 proliferation, while antiestrogenic compounds inhibit the proliferation in the presence of estradiol (Silva, Scholze, and Kortenkamp 2007).

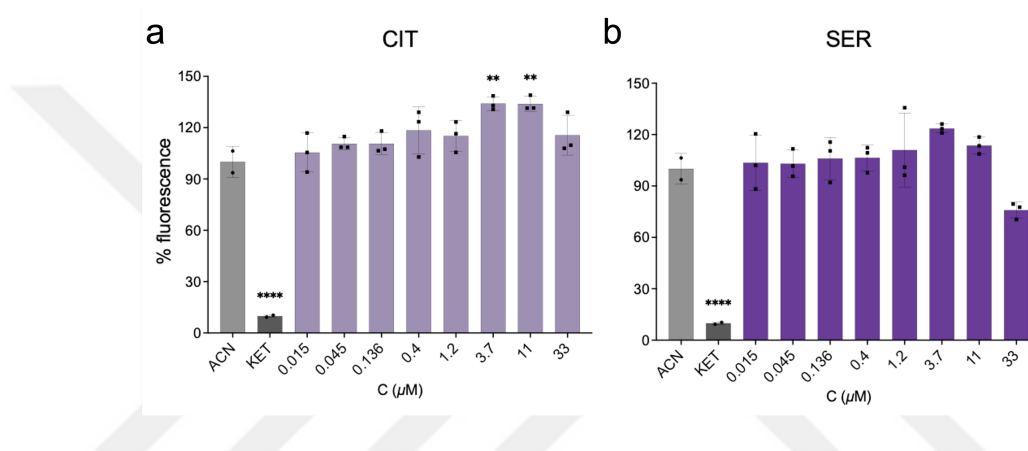
SSRIs did not increase MCF-7 cell proliferation (Fig. 4.20.a-b), nor decrease cell proliferation induced by 1 nM E2 (Fig. 4.20.c-d), indicating no agonistic or antagonistic activity on ER, confirming the results of reporter cell line experiments. Notably, SER decreased MCF-7 proliferation significantly at 11 and 33  $\mu$ M (Fig 4.20.b-d). However, this decrease was due to cytotoxicity (determined via visual inspections) rather than antiestrogenic activity.



**Figure 4.20** Effects on MCF-7 cell proliferation with (a) citalopram (CIT), and (b) sertraline (SER), determined via sulforhodamine B assay. Decrease in estrogen-induced MCF-7 proliferation with (c) CIT, and (d) SER. Bars represent means and error bars indicate standard deviations of 3 independent experiments. E2:  $\beta$ -Estradiol (1 nM). \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$  (GraphPad Prism, v9.0).

### 4.2.3. Aromatase Inhibition Assay

Aromatase inhibitory activity of the SSRIs was evaluated by the CYP19/MFC High-throughput Screening kit (Corning). CIT did not inhibit aromatase activity at the tested concentrations (Fig. 4.21.a), while SER showed aromatase inhibitory effects only at 33  $\mu$ M (Fig. 4.21.b). Significant activity was not observed around reported SSCs by either of the SSRIs.



**Figure 4.21** Aromatase inhibitory effects of citalopram (CIT) and sertraline (SER) evaluated by CYP19/MFC High-Throughput Screening kit. Results expressed as % fluorescence compared to vehicle control, acetonitrile (ACN). Bars represent means and error bars indicate standard deviations of triplicate measurements. KET: Ketoconazole (40  $\mu$ M). \*  $p < 0.05$ ; \*\*  $p < 0.005$  \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$  (GraphPad Prism, v9.0).

## 5. Discussion

In this thesis, the adipogenic potential of two commonly used SSRIs was investigated utilizing cell models to study adipocyte formation and nuclear receptor activation. This research is key, as these pharmaceuticals are associated with weight gain and could be potential contributors to the global increase in obesity. In this study, *in vitro* adipogenesis assays were used as a model for excessive body fat accumulation that is associated with obesity, and crucial mechanisms involved in adipocyte differentiation, such as activation of nuclear receptors like PPAR $\gamma$ , as well as several endocrine receptors were investigated. Results pointed toward disruption of adipocyte function rather than increased adipogenesis, which is an important outcome that should be followed up with further studies.

This discussion will first address the methodological strengths and weaknesses, focusing on the cell models for adipogenesis, methods to assess adipocyte differentiation, and the relevance of the tested concentrations in the cell model. Then, the obtained results will be discussed in the broader context of adipose tissue biology and function. Subsequently, the impact of this research on the scientific field will be discussed, before proposing recommendations for future research perspectives.

### 5.1. Methodological Strengths and Weaknesses

#### 5.1.1. Cell Models for Adipogenesis

There are several well-established cell models used to assess the effects on adipogenesis. These include MSCs or adipocyte progenitor cells, such as the murine 3T3-L1 cell line, that can be differentiated into mature adipocytes *in vitro* by exposing them to an adipogenic cocktail (Chen et al. 2016; Lustig et al. 2022). The well-characterized murine 3T3-L1 cell line remains the most frequently used cell model for adipogenesis in obesogen-related studies. However, it is important to note its limitations. Their sourcing can be unreliable, which contributes to the variability of results among different laboratories (Kassotis et al. 2021, 2022). Moreover, 3T3-L1 cells only allows the assessment of adipocyte differentiation, as these are adipogenic progenitor cells that are already committed to the adipogenic lineage. On the other hand, MSCs isolated from bone marrow remain multipotent, allowing for the

assessment of adipocyte lineage commitment in addition to adipocyte differentiation. As MSCs can be obtained from human donors, donor-to-donor differences contribute to the high variance seen among different batches of cells. Nevertheless, the results with them can be better translated to humans which provides an important advantage over non-human cell lines in eliminating problems arising from interspecies differences (Kassotis et al. 2022; Legler et al. 2020).

Studies utilizing these cell models mostly rely on 2D monolayer cultures for their simplicity and cost-effectiveness (Legler et al. 2020). However, there are significant differences in morphology, size, and transcriptional profiles when comparing 2D-grown adipocytes to *in vivo* adipose tissue (Klingelhutz et al. 2018). Recently, 3D spheroid models have been developed to improve adipogenic testing, and studies with murine and human preadipocytes have shown 3D adipogenesis models to be more representative of *in vivo* conditions with improved differentiation and gene expression (Klingelhutz et al. 2018; Muller et al. 2019; Shen et al. 2021). Although these spheroid models show great improvements over conventional 2D models, they were all developed by using adipocyte progenitor cells.

In our study, MSCs were employed in a 3D set-up with the purpose of improving the model even further by enhancing both human relevance and model applicability. First, the seeding density and differentiation conditions were optimized for the 3D model. 5000 cells per spheroid were found to be optimal for spheroid growth and differentiation, an amount also used in other studies (Shen et al. 2021). One of the main concerns in 3D cell models is the formation of a hypoxic core, due to decreased oxygen diffusion towards the center of the spheroid (Trayhurn et al. 2008). However, both histological analyses using Hematoxylin & Eosin (H&E) staining and confocal imaging confirmed an even distribution of lipid droplets throughout the spheroid without any sign of a necrotic core. Likely, the limited number of cells allowing rapid diffusion of nutrients and oxygen aided in this observation, compared to previous studies with higher cell numbers (Schmitz et al. 2021).

To characterize the 3D model, the prototypical *PPAR $\gamma$*  agonist ROSI was used to compare 3D spheroids to 2D-grown adipocytes for differences in phenotype and transcriptional profiles. Differences between the 2D and 3D models were found based on lipid droplet phenotype and morphology, as well as on the transcriptome. Spheroids exposed to ROSI differed from 2D adipocytes in phenotype following differentiation,

forming fewer and larger lipid droplets that more closely resemble *in vivo* adipose tissue. This was also shown in multiple publications, as well as transcriptomic profiles that resemble the human situation better than the 2D models (Klingelhutz et al. 2018; Shen et al. 2021).

Novel omic technologies have enabled a more in-depth investigation of the complex transcriptomic profile of *in vitro* differentiated adipocytes. RNA-seq in 2D and 3D models followed by pathway analysis revealed quite comparable results, showing metabolic pathways were affected similarly after ROSI treatment in both models. Notably, more pathways related to insulin and adipocytokine signaling and structural integrity were found altered in the 3D model. Within the same treatment, most pathways were downregulated in 3D compared to 2D, except the ribosome pathway, which was strongly upregulated in the 3D model, as shown in a recent study (Shen et al. 2021). The TGF-beta signaling pathway, known to inhibit adipogenesis by reducing *PPAR $\gamma$*  expression and *CEBP $\beta$*  activation (Chen et al. 2016), was strongly downregulated in the 3D model. This stronger inhibition in 3D might lead to increased adipogenesis, by providing a more suitable setting for the induction of adipogenic genes, as shown in a previous study (Shen et al. 2021).

Next, a set of obesogens were tested in parallel experiments and the 2D and 3D responses were compared to assess the performance of the 3D model. A similar response with the obesogens was observed between the two models, however gene expression generated unexpected results. QPCR analysis revealed significant differences in gene expression profiles between the different models. ROSI and other suspected *PPAR $\gamma$*  agonists led to similar expression of adipogenic genes in both models, whereas the differences were more pronounced between the models for the obesogens acting through other mechanisms. These differences might indicate that the 3D model is more sensitive to obesogens acting via mechanisms other than *PPAR $\gamma$*  activation, which may be concealed in the 2D model but apparent in the more complex 3D context.

Overall, the 3D model showed a more similar phenotype to adipose tissue and transcriptional profiles indicate a different response to adipogenic stimuli than 2D, supporting the findings of previous publications (Klingelhutz et al. 2018; Shen et al. 2021). The benchmark dose (BMD) approach confirmed that the adipogenic potencies



of the models were comparable, indicating the novel 3D model's applicability for screening chemicals' effects on adipogenesis.

### **5.1.2. Methods to Assess Adipocyte Differentiation**

Increased lipid storage within cells is often used as a measure of enhanced adipogenesis in cell models. A variety of lipid stains were developed to characterize the effects of chemicals on lipid accumulation including Oil Red O, Nile Red, and Bodipy 493/503. Oil Red O and Bodipy can be used to stain neutral lipids, whereas Nile Red has an affinity for both neutral and phospholipids and can be used to detect both (Fam, Klymchenko, and Collot 2018).

Multiple staining procedures were used in MSCs for the screening of SSRIs (see methods). After Nile Red staining, green-fluorescent signal was measured for neutral lipids by using a fluorescence plate reader and by high-throughput imaging for the 2D and 3D models, respectively. High-throughput imaging was found to be an efficient method to analyze 3D spheroids. However, plate reader analysis for the 2D model proved to be challenging due to the different phenotypes of lipid droplets induced by the SSRIs. When 2D adipocytes were inspected under a fluorescence microscope, it was apparent that positive control ROSI had bigger adipocytes with bigger lipid droplets, compared to CIT and SER which seemed to have adipocytes that were smaller in size and filled with smaller lipid droplets. While the increase in lipid droplets within cells could be observed by fluorescence microscopy, it was difficult to quantitatively measure this effect with the plate reader. Flow cytometry analysis showed higher sensitivity, thus we were able to quantitatively measure the induction of adipogenesis and better determine the obesogenic potential of CIT and SER. Moreover, Nile Red staining allowed for the assessment of different lipids (by measuring green- and red-fluorescent signals for neutral and phospholipids, respectively) which aided in the analyses of different lipid profiles induced by the SSRIs in follow-up experiments.

In the additional MSCs experiments (2D), Bodipy staining was used in combination with LysoTracker Red to assess the effects on neutral lipids and lysosomes during adipocyte differentiation. Bodipy was selected due to the lack of interference between its green-fluorescent signal and the red-fluorescent signal produced by LysoTracker.

Additionally, Bodipy can be used for live staining which was needed for our experiments as LysoTracker stains require live staining to detect and trace lysosomes. Using the plate reader, we were able to quantitatively measure the effects on neutral lipids and lysosomes. On the other hand, fluorescence imaging was more difficult due to Bodipy's high background signal and limited photostability.

In the 3T3-L1 experiments, Oil Red O, a lipid-soluble diazolic dye that is selective for neutral lipids, was used. A disadvantage of Oil Red O staining over Nile Red and Bodipy is its poor solubility, thus an extra step of dissolution of the dye in isopropyl alcohol is needed prior to plate reader analysis. Although the cells are fixed, the use of such invasive solvents could cause the disruption of lipid droplets within the cells, which could potentially lead to the wrong estimation of lipid accumulation and adipocyte differentiation.

Overall, Nile Red staining followed by high-throughput imaging (3D) or flow cytometry analysis (2D) were proven to be the best methods for our experimental needs. Bodipy staining could also be a valuable tool depending on the experimental setup, for its compatibility with other dyes. In future studies, the use of high content imaging with Nile Red staining would be beneficial in the 2D model, as it would also allow for the assessment of lipid droplet phenotype and distribution.

### **5.1.3. Relevance of Tested Concentrations**

The effects observed in our study are remarkably close to the steady-state concentrations observed in clinical studies. These concentrations represent the total concentration of the drug in plasma, which includes both the protein-bound and unbound (free) fractions. Notably, around 80 % of CIT is bound to plasma proteins, while the ratio for SER is between 95-99 % (DeVane 1999; Pollock 2001). They are both distributed extensively into various organs and tissues in the body, reflected by a large distribution volume (Baumann 1992; De Vane et al. 2002), and due to their lipophilic nature, they can be expected to reach higher concentrations in the adipose tissue. A recent publication on a physiologically based pharmacokinetic model for CIT estimates that levels in adipose tissue might be more than three times higher than in plasma (Wu et al. 2020). In short, despite the high protein binding of these

compounds, *in vitro* concentrations at which the effects were observed reflect the levels seen in humans.

It is plausible that drugs binding to plasma proteins might also bind to proteins present in the FBS, thus lowering their availability in the cell model. Because our system includes proteins from FBS, we cannot precisely know the availability of SSRIs to the MSCs. It's challenging to precisely determine the concentration of the unbound fraction of the drug in the cell model, as we lack knowledge of the chemicals' kinetics in this system and the extent of binding to FBS proteins may differ from that in plasma. Analytical measurement of such chemicals within cells would give the right figures on what is actually taken up by the cells. In this study, as the unbound fraction might be less than the nominal concentrations added to the system, there is a possibility that BMD values might be an underestimation.

All in all, there is an uncertainty in exposure levels of the bioavailable fraction, and further research into the model, including chemical analysis of such pharmaceuticals in the *in vitro* system, is warranted for *in vitro* to *in vivo* extrapolation. Additionally, reducing the FBS concentration in cell culture medium or optimizing serum-free conditions in future studies could potentially counter this shortcoming of the model.

## **5.2. General Discussion of the Results**

The results presented here aid in the elucidation of the adipogenic potential and the underlying mechanisms of the SSRIs. The effects on adipocyte differentiation were studied by using human MSCs and the murine 3T3-L1 cell line, mainly focusing on MSCs for a comprehensive assessment, and looking into possible mechanisms involved in adipocyte differentiation.

While assessing the adipogenic potential of CIT and SER, initially, an increase in lipid accumulation was observed in MSCs, an indication of enhanced adipogenesis. This effect was observed in a concentration-dependent manner, and using the BMD approach, points of departure were found to overlap with reported SSCs (SER: 0.065-0.65  $\mu$ M, and CIT: 0.12-0.92  $\mu$ M) (Baumann 1996; De Vane et al. 2002). Fluorescence and confocal microscopy of 2D MSCs showed a distinct phenotype of small lipid

droplets with the SSRIs compared to ROSI, which led us to question whether this could be induced by a different mode of action.

There are multiple mechanisms through which chemicals can induce adipocyte differentiation. This study focused on the mechanisms involving key nuclear receptors regulating adipogenesis, to elucidate the underlying mechanism behind SSRIs' effects. First, the agonistic activity on *PPAR* $\gamma$ , the key regulator of adipogenesis, and *PPAR* $\alpha$  was explored by using receptor binding and transactivation assays. However, agonistic activity was not observed with CIT and SER, on either *PPAR* $\gamma$  or *PPAR* $\alpha$ .

In animal studies prenatal exposure to estrogenic obesogens has been shown to promote the development of obesity in the offspring, possibly occurring through estrogen receptor activation (Darbre, 2017; Heindel et al., 2022; Newbold et al., 2007), whereas reduced androgen levels and anti-androgenic therapy has been associated with decreased adiposity (Kassotis et al. 2017). Additionally, some obesogens with dioxin-like activity have been shown to induce adipogenesis via indirectly altering *PPAR* $\gamma$  expression (Casals-Casas and Desvergne, 2011). Therefore, the estrogenic, androgenic, and dioxin-like effects were explored by using receptor binding and transactivation assays for the ER, AR, and DR, along with ER-dependent proliferation and cell-free aromatase inhibition (the enzyme responsible for estrogen synthesis) assays. Our results revealed SSRIs' modulatory effects on adipogenesis were not due to estrogenic, androgenic, or dioxin-like activity, nor significant aromatase inhibitory activity. CIT and SER were previously shown to inhibit aromatase activity in two microsome-based *in vitro* assays (Jacobsen et al. 2015). Although the extent of inhibition varied between the two assays, the results indicate an influence on the enzyme by both SSRIs. Notably, the concentrations tested in that study covered a wide range. Therefore, the reported effects occurred at higher concentrations than those tested in this study, with reported IC<sub>50</sub> values of 55.8 and 90.6  $\mu$ M for CIT and SER, respectively. This difference in concentration levels might explain the absence of aromatase inhibition with the SSRIs in our experiments. Nevertheless, our results indicate a mechanism beyond endocrine modulation driving the adipogenic effects.

RNA-seq revealed a distinct gene expression profile in 3D after SSRI treatments compared to positive control, ROSI. To our surprise, key adipogenic genes and important pathways related to adipocyte differentiation (such as PPAR signaling,

adipogenesis, and fatty acid metabolism) were found downregulated compared to vehicle control (DMSO). This is supported by the QPCR analysis, where key regulators of adipogenesis *PPAR $\gamma$*  and *CEBP $\alpha$* , and genes involved in PPAR signaling *FABP4* and *ADIPOQ*, were found downregulated by the SSRI treatments.

These findings were repeated in the additional 3T3-L1 experiments, by an observed decrease in adipogenic protein expression, especially with SER. Conversely, CIT was found to slightly induce *PPAR $\gamma$*  and *CEBP $\alpha$*  protein levels, yet the lack of increase in lipid accumulation suggests this was not sufficient to induce adipocyte differentiation. Nevertheless, altered expression of key adipogenic proteins by the SSRIs indicates their potential impact on adipocyte development and function.

When the RNA-seq data was examined for other pathways affected by the SSRI treatments, pathways related to phospholipids and lysosomes were found upregulated by all exposures. Phagosome, and phospholipase D, other pathways that can be related to lysosome function, were significantly affected by CIT at the highest concentration (10  $\mu$ M). These findings were confirmed with additional experiments in 2D MSCs by specific staining, where an induction was observed for both phospholipids and lysosomes. Interestingly, in these experiments, the induction in phospholipids was higher compared to neutral lipids in the same model, showing even lower BMD levels as with neutral lipids. Moreover, for SER the induction in phospholipids was observed in the range of reported SSCs.

Next, additional experiments were performed on mature adipocytes to follow up on these effects. The effects of CIT and SER on neutral lipids and phospholipids after differentiation were studied, to see whether the SSRIs would change the lipid profile from neutral lipids to phospholipids. As expected, there was no significant effect on neutral lipids by the SSRI treatments after differentiation. However, phospholipid levels increased, implying the effects of SSRIs are also apparent in mature adipocytes. This could have clinical implications, as the patients receiving antidepressant treatment are mostly adults.

All our findings together led us to the conclusion that these SSRIs lead to a common adverse effect of a group of chemicals, commonly known as cationic amphiphilic drugs (CADs). These chemicals are known to accumulate within lysosomes due to their weak basic properties. CIT and SER share the chemical structure of CADs which

features a protonable amine group attached to a lipophilic component. This lipophilic nature allows the unprotonated neutral form to pass through cellular membranes. However, upon reaching an acidic environment, such as lysosomes, the amine group is protonated and the positively charged form is no longer able to pass through the membrane. Consequently, the compound becomes “trapped” and accumulates within the lysosomes (Kazmi et al., 2013). Lysosomal trapping via this mechanism results in extensive binding to phospholipid membranes and the inhibition of lysosomal enzymes, specifically lysosomal phospholipase A2 (LPLA2). Located on the lysosomal membrane, LPLA2 is mainly involved in the degradation of lysosomal phospholipids (Hinkovska-Galcheva et al., 2021). Lysosomal accumulation of CADs can result in competitive inhibition of LPLA2, leading to excessive accumulation of lysosomal phospholipids, a process known as drug-induced phospholipidosis. This phenomenon is mainly shown *in vivo*, with lung and liver being common targets, ultimately leading to pulmonary or liver fibrosis (Hinkovska-Galcheva et al., 2021; Reasor et al., 2006).

Considering the existing knowledge on the physiochemical properties of CIT and SER (Reasor et al. 2006), and that both SSRIs were reported to inhibit LPLA2 with a reported IC<sub>50</sub> of 8.6 and 19.5  $\mu$ M, respectively (Hinkovska-Galcheva et al. 2021), our results strongly point towards their lysosomal accumulation in maturing adipocytes, inhibiting LPLA2 to promote phospholipid accumulation. The current gene expression data does not show a difference in LPLA2 expression after SSRI treatments. However, this is not surprising as the inhibition of LPLA2 occurs through a molecular mechanism that is not regulated by gene expression.

A recent study tested the effects of some CADs (not including CIT or SER, but including amiodarone and another SSRI, fluoxetine) on 3T3-L1 cells and reported a positive correlation between lysosomal accumulation of CADs and decreased adipocyte differentiation (Kagebeck et al. 2018). They concluded that high accumulation of CADs in lysosomes leads to lysosomal dysfunction and the inhibition of autophagy, the process of lysosomal degradation of intracellular components like damaged organelles or proteins (Cabrera-Reyes et al. 2021), which in turn inhibits adipocyte differentiation. Their findings align with our results of SSRIs’ inhibitory effects on adipogenesis in MSCs and 3T3-L1 cells.

Another study with the 3T3-L1 cell line showed high accumulation of CADs in mature adipocytes (after differentiation) via binding to phospholipid membranes (Sanchez Garcia et al. 2018), which confirms the results of our experiments with MSCs in the mature adipocyte setup. This suggests that CADs binding to phospholipid membranes, which is a fundamental step in lysosomal accumulation shown across various cell types, is likely occurring in differentiating and mature adipocytes.

Finally, the downregulation of many adipocyte-related pathways made us hypothesize whether SSRIs were antagonists for *PPAR* $\gamma$ . However, additional experiments on the *PPAR* $\gamma$  reporter using an antagonist set-up did not show any effect. The process of *PPAR* $\gamma$  activation during the initial step of adipogenesis involves the release of arachidonic acid from membrane phospholipids. Cytosolic phospholipase A2 (cPLA2) is a critical enzyme in arachidonic acid mobilization and subsequent prostaglandin (PG) production. Specifically, PG15d acts as an endogenous ligand for *PPAR* $\gamma$ , initiating the expression of adipogenic genes. In this context, cPLA2 acts as a proadipogenic factor in early adipocyte differentiation. Recent studies emphasized the importance of the phospholipase A2 pathway in adipocyte differentiation (Peña et al. 2016). In previous studies, azithromycin, a known CAD, was shown to inhibit PG synthesis, likely due to high binding to the phospholipid membrane and subsequent disruption of cPLA2 activity (Banjanac et al. 2012). It is likely that the positive correlation between CAD accumulation and the inhibition of adipocyte differentiation could be a result of the inhibition of the signaling cascade leading to *PPAR* $\gamma$  activation (Kagebeck et al. 2018). Although, we do not know exactly how these SSRIs are able to suppress adipogenesis in MSCs, our hypothesis is that the same mechanism could be underlying this effect, along with increased production of phospholipids and a balance towards lysosome formation, leading to less functional adipocytes.

### **5.3.Impact of Research for the Scientific Field and Future Perspectives**

Currently, the efforts in the obesogen field are focused on developing new human-relevant models to identify potential obesogens (Kassotis et al. 2022; Legler et al. 2020). The novel 3D model we employed for the assessment of SSRIs offers a robust platform for assessing adipogenesis and screening obesogens. Our results with the 3D model demonstrate its ability to reveal novel mechanisms involved in obesogen action,

otherwise not observed in 2D. Although a direct comparison to human adipose tissue in terms of transcriptional profiles would be the next step to characterize this model, all our analyses indicate it a suitable model for assessing the effects of potential obesogens on adipogenesis. In future studies, the model could be further improved by the inclusion of additional cell lines found in adipose tissue, such as macrophages and endothelial cells, which would enhance physiological relevance and shed light on the interplay between metabolic organs and immune responses. Nevertheless, the 3D model holds promise in replacing conventional 2D methods with preadipocyte cell lines, moving obesogen research even further by answering the need for better and more human-relevant testing methods.

The current approach in the obesogen field is to assess whether a chemical can be classified as an obesogen, by using a weight-of-evidence approach. For this approach, it is crucial to identify molecular initiating events and key events to generate an Adverse Outcome Pathway (AOP) for metabolism disruption (Legler et al. 2020). Although there is no complete AOP for metabolism disruption or obesity, this study indicates lysosomal disruption as a key event for drug-induced adipocyte dysfunction and paves the way for future research directions. Furthermore, AOPs have been developed around lysosomal disruption for liver toxicity (AOP144, lysosome dysfunction; AOP130, phospholipase inhibition) (Kuburic et al. 2023; Oh et al. 2023), in which many key events are present in our data. Apart from lysosome formation, mitochondrial dysfunction is a common key event in both AOPs, similarly, mitochondria function (Oxidative Phosphorylation) was also predicted to be affected in our RNA-seq dataset. CADs are also known to accumulate in mitochondria, causing increased proton transfer across the inner mitochondrial membrane towards the matrix, which in turn disrupts electron transfer crucial for ATP synthesis (Fromenty, 2023). Future research with chronic exposure experiments might give insights into the further progression of this pathway leading to inflammation and cell death, which would be an important step in developing an AOP for metabolism disruption with these pharmaceuticals.

Our assessment of CIT and SER strongly points toward their ability to disrupt important adipogenic processes *in vitro* and pave the way for future research directions. To our knowledge, there are no reported effects on adipogenesis from *in vivo* studies following CIT or SER exposure. Future studies in more complex animal



models should focus on the (developmental) effects of CIT and SER, specifically related to metabolism and associated endpoints such as dyslipidemia, inflammation, and fibrosis in adipose tissue, as well as mechanistic experiments into the role of LPLA2 in adipocytes.

Given the common use of CIT and SER during pregnancy, it is important to explore whether these effects extend into later stages of life due to exposure during fetal development. Epidemiological studies to date mainly focus on fetal and neonatal outcomes of prenatal SSRI exposure (Fitton et al. 2020; Lebin and Novick 2022). Because of the lack of longitudinal studies exploring metabolic outcomes, our knowledge of the risks of maternal SSRI use related to obesity and metabolism is limited. Exploration of these areas by long-term studies that follow up children after birth and throughout childhood, or even into adulthood should be the focus of future epidemiological studies. Such studies would enhance our understanding of the relationship between prenatal exposures and potential health outcomes in children, contributing to future safety assessments of SSRIs.

## 6. Conclusion

The rise in obesity calls for the exploration of chemicals' influence, including pharmaceuticals, on adipose tissue development and function to promote obesity. The growing fetus can also be exposed to these obesogens through the placenta, which increases obesity and related metabolic disease risk in adulthood. Antidepressants, particularly SSRIs, stand out among the diverse range of pharmaceuticals for their high prescription rates, despite substantial evidence pointing towards their potential impact on metabolism and weight gain. Considering their common use during pregnancy, these effects might extend into the later stages of life due to exposure during fetal development.

This thesis increases our understanding of how CIT and SER affect the process of adipocyte differentiation and provides valuable insights into the mechanisms behind their action. Our findings reveal the profound impact of CIT and SER on lipid metabolism within differentiating and mature adipocytes in the range of reported steady-state plasma concentrations. Contrary to existing epidemiological studies that associate long-term treatment with these compounds with weight gain, our results paradoxically unveil a counterintuitive suppression of adipogenesis on the basis of gene expression. Transcriptomic profiling proved to be a valuable tool in discovering undetected mechanisms, in combination with whole transcriptomics and functional analyses in MSCs, we were able to pinpoint the mode of action of these SSRIs, surprisingly pointing towards the formation of lysosomes and increased phospholipids instead of neutral lipids, indicating the formation of dysfunctional adipocytes.

In conclusion, our data strongly suggest potential implications for the maintenance of a physiologically balanced metabolism. Although the systemic consequence of these effects is complex and difficult to predict, our results are an important step in further evaluating the safety of these SSRIs on endpoints that have not been studied extensively. The results of this comprehensive research offer valuable insights into SSRIs' potential impact on adipogenesis and their part in the complex landscape of obesity. Thereby, underscore the need for heightened caution when employing these pharmaceuticals, particularly during pregnancy.

## References

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## Acknowledgements

I always knew I wanted to pursue a doctorate degree. Well maybe not always, a PhD is hardly someone's dream as a child, but when I started studying at the Faculty of Pharmacy, doing research was something in the back of my mind. This idea grew over the years of my study, and I finally found myself in the PhD program of the Dept. of Pharmaceutical Toxicology. Looking back at my journey today, I can happily say that I am very glad I chose this path. It has been a wild journey with lots of ups and downs, yet I cannot imagine myself doing anything else where I would have learned as much as I did and grew both as a person and as a scientist. For this I have to thank a few people, who helped me become the scientist I am today.

First of all, **Prof. Dr. Hande Gürer Orhan**, I am very lucky to have you as my supervisor. Meeting you during my bachelor studies was surely one of the turning points in my life. Working together for over five years now, I can say without a doubt that you are a wonderful person and a great mentor to look up to. Thank you for always being there for me, helping me overcome all kinds of problems, and sending me off to a new adventure, believing in me more than I believed in myself!

Which brings me to the next person I am very grateful for, **Ass. Prof. Dr. Jorke Kamstra. Jorke**, our collaboration was most certainly another turning point in my life. Coming to the Netherlands for a 12-month project was undoubtedly something way out of my comfort zone, little did I know it would be a very fruitful partnership that will be extended to a total of 19 months! (Felt like it was still too short, if you ask me:) You and **Mariska** made me feel like at home, and I will forever be grateful for your kindness and generosity. Thank you for everything you've taught me, in and outside the lab, for trusting me and giving me the opportunity to be a part of the team. It was an absolute pleasure working with you and learning from you. Needless to say, this dissertation would not be possible without your guidance and contributions.

I would like to thank the faculty members of the Dept. of Pharmaceutical Toxicology, **Prof. Dr. Hilmi Orhan, Prof. Dr. Ferzan Lermioğlu Erciyas, and Ass. Prof. Dr. Sumru Sözer Karadağlı** for all their guidance and support during my PhD studies.

I also want to thank my dear friends from the department **Nina and Elif**, whose friendships extend beyond the lab (and beyond borders:), as well as **Ege, Kemal, Şadiye, Batuhan, and Rasih**, for all their help and support.

I would like to thank **Prof. Dr. Juliette Legler**, for agreeing to our collaboration as the head of the Institute for Risk Assessment Sciences (IRAS), which provided me with a wonderful opportunity. I am also very grateful to all the members of IRAS for the warm welcome into their department, especially **Annemijne, Emma, Lennart, Ivo, Jack, Roeland, Jiayi, Martina, and Daniela** for the great office vibes and all the amazing outings. A very special thanks to **Sandra Nijmeijer, Nikita Korpel, Sander Lentz, Rik van Dellen, and Jeroen van Voorthuizen** with whom I collaborated on some parts of my thesis and formed valuable friendships along the way.

A huge thank you to my lovely ladies **Hania, Susana, Lora, and Margherita** for welcoming me with open arms and being your unique selves. I could not have wished for better friends than you, chicas! Thank you for all the true-crime evenings, game night sessions, pub crawls, garden parties, BBQs in the park, the absolute craziness of King's Day, and the epic midweek madnenses! The same goes for **Jordi, Hannes, Sjors, and Tiago**, it still shocks me that I got to meet you crazy people. Thank you for all the amazing memories, and cheers to many more!

A very special thank you to my dearest friends, **Gökselin, Elif, Nehir, and Yağmur**, for always being by my side, making me laugh and keeping me sane through the most stressful times. Your friendship means the world to me, and I feel incredibly lucky to have friends like you!

Last but not least, I would like to thank my incredible family for supporting me through this journey, even across borders. **Defne**, thank you for always being there to hold my hand when I was upset and to share my joy when I was happy. **Mom and Dad**, you are my inspiration, always. I hope I managed to (and will continue to) make you proud. Thank you for your unending love and support, I love you!

Research at Ege University for this dissertation was supported by BAP project under grant no. TDK-2022-23558 and by TUBITAK 1001 Project under grant no. 121S657. A fellowship at Utrecht University for 12-months was granted by TUBITAK 2214-A-International Doctoral Research Fellowship Program. Research at Utrecht University was supported by DGK Jubileumfonds and by European Union's Horizon 2020 research and innovation program GOLIATH under grant agreement no. 825489.

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### ***Technical Expertise***

Experienced in cell culture research (stem cell culture and differentiation, primary and immortalized cell culture, 2D monolayer and 3D organoid culture), cell-based functional assays (cell proliferation, cell death, fluorescence/luminescence measurements).

Experienced in *in vitro* assays, mainly for the assessment of endocrine/metabolism disrupting compounds.

Hands on experience in molecular biology methods including ELISA receptor binding, quantitative polymerase chain reaction (QPCR), and flow cytometry.

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Experienced in Microsoft Office and GraphPad (data analysis), SeqMonk sequence analysis tool (RNA-sequencing), ClustVis and Cytoscape web tools (visualization of RNA-seq data), BD Accuri C6 software (flow cytometry), Tecan i-control, Image J, and CellProfiler softwares (microscopy image analysis).

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2. Entezari B, **Bozdağ D**, and Gürer Orhan H. 2023. “Evaluation of Endocrine Related Adverse Effects of Non-Endocrine Targeted Pharmaceuticals in Cellular Systems” *Endocrine, Metabolic & Immune Disorders - Drug Targets* 23(14):1740–1749. doi: 10.2174/1871530323666230224145624.
3. Entezari B, **Bozdağ D**, and Gürer Orhan H. 2022. “Obesogens: Definition, Mechanisms of Action, Potential Industrial Chemicals and Pharmaceuticals” *Istanbul Journal of Pharmacy* 52(2):215–225. doi: 10.26650/istanbuljpharm.2022.1027027.

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2. International Congress of Toxicology (ICT) Meeting, Maastricht (NL), 2022.  
**Bozdag D**, Gürer Orhan H, and Kamstra J.H. “*In vitro* investigation of potential endocrine disrupting and obesogenic effects of sertraline and citalopram”
3. Annual EURION Cluster Meeting, Amsterdam (NL), 2023.

**Bozdag D**, Gürer Orhan H, and Kamstra J.H. “*In vitro* investigation of potential endocrine disrupting and obesogenic effects of sertraline and citalopram”

4. Annual NVT Meeting, Ede (NL), 2023.

**Bozdag D**, van Voorthuizen J, and Kamstra J.H. “Cationic amphiphilic drug induced phospholipidosis and lysosomal dysgenesis during *in vitro* adipogenesis”

#### ***Awards and Scholarships***

1. Deniz Bozdağ has received the award for **best oral presentation** at the Annual Meeting of the Dutch Society of Toxicology (NVT), Ede (NL), 2023.
2. The project “*In vitro* Investigation of Potential Endocrine Disrupting and Obesogenic Effects of Sertraline and Citalopram” was sponsored by The Scientific and Technological Research Council of Turkey (TÜBİTAK)’s 2214-A International Research Fellowship Program for PhD Students (04/2022-04/2023)

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