THE EFFECTS OF GREEN TEA AND POMEGRANATE POLYPHENOLS ON IN VITRO AND IN VIVO VEGF ACTIVITY

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Abstract

Background: The green tea and pomegranate polyphenols epigallocatechin gallate (EGCG) and ellagic acid (EA) have previously demonstrated anti-atherosclerotic effects and inhibition of VEGFR-2 phosphorylation in cell models. Vascular endothelial growth factor (VEGF) injection into animal models induced atherosclerosis. This thesis aims to investigate whether polyphenols which potently inhibit VEGF-induced VEGFR-2 phosphorylation at physiological concentrations *in vitro* will be able to inhibit VEGF-induced atherosclerotic development *in vivo*.

Methods: The inhibition of VEGF-induced VEGFR-2 phosphorylation in Human umbilical vein endothelial cells (HUVECs) by EGCG, punicalagin, EA and the urolithins was investigated. The effect of 1.8 g/kg EGCG and EA dietary supplementation on VEGF-induced atherosclerosis was investigated in the ApoE-/- mouse model.

Results: EGCG, EA and punicalagin inhibited 50 % VEGF activity, quantified by VEGFR-2 phosphorylation in HUVECs, at 96, 310, and 49 nM. EA metabolism reduced the VEGF inhibitory activity. EGCG and EA significantly inhibited VEGFR-2 phosphorylation at physiological concentrations, predominantly through interactions with the VEGF ligand. 3 weekly intraperitoneal injections of VEGF into mice did not induce atherosclerosis. EGCG and EA dietary supplementation did not affect plaque size but induced small increases in circulating LDL and total cholesterol compared to controls. EGCG and EA injection reduced plaque thickness but not overall size. It was shown that foetal calf serum reduced the polyphenol-mediated inhibition of VEGF activity.

Conclusions: The data in this thesis suggest that (1) multiple injections of VEGF does not induce atherosclerotic plaque growth in the ApoE-/- mouse model under the study conditions, (2) a mixture of EGCG and EA had no significant effect on the growth of atherosclerotic plaques when supplemented into the diet of $ApoE^{-/-}$ mice, and (3) serum proteins limit the ability of polyphenols to inhibit VEGFR-2 phosphorylation. These results therefore suggest it is unlikely EGCG and EA reduce atherosclerosis by inhibiting the VEGF ligand *in vivo*.

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Abbreviations

AKT	Protein kinase B
ApoE-/-	Apolipoprotein E double knockout
Axl	Tyrosine-protein kinase receptor UFO
BAECs	Bovine aortic endothelial cells
BCA	Bicinchoninic acid
B-Raf	B-Raf proto-oncogene
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
Ca^{2+}	Calcium
C-ABL	Abelson murine leukemia viral oncogene homolog 1
C_{max}	Maximal plasma concentration
COMT	Catechol-o-methyltransferase
CVD	Cardiovascular disease
DAD	Diode-array detection
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's media
EA	Ellagic acid
EC	Epicatechin
ECG	Epicatechin gallate
ECM	Extracellular matrix
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
eNOS	Endothelial nitric oxide synthase
FAK	Focal adhesion kinase
FCS	Foetal calf serum
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
HDL	High density lipoprotein
HIF1α	Hypoxia inducible factor 1 alpha
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

HSA	Human serum albumin
HSP90	Heat shock protein 90
HUVECs	Human umbilical vein endothelial cells
Grb2	Growth factor receptor-bound protein 2
IDL	Intermediate density lipoprotein
IP_3	Inostinol-3-phosphate
I.P.	Intraperitoneal
IPR	IP3 receptor
IQGAP1	Ras GTPase-activating-like protein IQGAP1
I.V.	Intravenous
LCMS	Liquid-chromatography mass spectrometry
LDL	Low density lipoprotein
LDLR-/-	Low density lipoprotein receptor double knockout
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinases
MSA	Mouse serum albumin
NaPi	Sodium phosphate buffer
Nck-1	NCK adaptor protein 1
NO	Nitric oxide
NRP	Neuropilin co-receptor
PAF	Platelet-activating factor
PAK-2	p21-activated kinase 2
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDIA3	Protein disulfide isomerase isoformA3
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
Plc-γ	Phospholipase C gamma
PMSF	Phenylmethanesulfonyl fluoride
Puni.	Punicalagin
Raf	Rapidly accelerated fibrosarcoma

ROCK	RhoA kinase
ROS	Reactive oxygen species
SAPK2	Serine/threonine-protein kinase SAPK2
Sck	Shc-related adaptor protein
Shb	SH2 domain-containing adapter protein B
Src	Proto-oncogene tyrosine-protein kinase Src
SULT	Sulfotransferases
TMB	3, 3’, 5, 5’-Tetramethylbenzidine
TSAd	T-cell specific adapter protein
UGT	Uridine 5'-diphospho-glucuronosyltransferase
Uro	Urolithin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vLDL	Very low density lipoprotein
WHO	World Health Organisation

Symbols

CO_2	Carbon dioxide
cm_2	Centimetre squared
g	Gram
h	Hour
kDa	Kilodalton
kg	kilogram
mg	Milligram
ml	millilitre
mM	Millimolar
ng	Nanogram
nM	nanomolar
nmol/L	Nanomole per litre
v:v	volume per volume
Y	Tyrosine
µg	Microgram
µm	Micrometre
μm^2	Micrometre squared
µM	Micromolar
µl	Microlitre
°C	Degrees Celsius
%	Percent

List of publications

Research Journals

R.L. Edwards, P. Needs, S. Robinson, F.A. Tomás Barberán, P.A. Kroon, Can dietary polyphenols prevent cardiovascular disease by inhibiting vegf-dependent angiogenesis in atherosclerosis?, Atherosclerosis, Volume 235, Issue 2, August 2014, Page e95, ISSN 0021-9150

Edwards, R. L. and Kroon, P. A. (2014) Inhibition of VEGF Signaling by Polyphenols in Relation to Atherosclerosis and Cardiovascular Disease, in Recent Advances in Polyphenol Research, Volume 4 (eds A. Romani, V. Lattanzio and S. Quideau), John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9781118329634.ch9

In preparation: Edwards RL, Vauzour D, Kroon PA. Three consecutive weekly injections of VEGFA does not increase the size of aortic sinus atherosclerotic plaques in ApoE$^{-/-}$ mice on an atherogenic diet. Target journal: *Atherosclerosis.*

In preparation: Edwards RL, Vauzour D, Garcia-Villalba R, Tomas-Barberan FA, Kroon PA. 6 weeks dietary supplementation with epigallocatechin gallate and ellagic acid in ApoE$^{-/-}$ mice fed an atherogenic diet does not reduce atherosclerotic plaque size or circulating cholesterol concentrations. Target journal: *The Journal of Nutrition.*

In preparation: Edwards RL, Needs PW, Tomas-Barberan FA, Kroon PA. Comparison of the inhibition of VEGF-induced VEGFR2 activation by punicalagin and ellagic acid with their urolithin microbial metabolites and human phase-2 conjugates. Target journal: *Molecular Nutrition and Food Research.*

Posters

Rebecca L. Edwards, Paul Needs, Stephen Robinson, Francisco A. Tomás Barberán, Paul A. Kroon. Can dietary polyphenols prevent cardiovascular disease by inhibiting VEGF-dependent angiogenesis in atherosclerosis? *IFR Student Science Showcase,* June 2014.

Rebecca L. Edwards, Paul Needs, Stephen Robinson, Francisco A. Tomás Barberán, Paul A. Kroon. Can dietary polyphenols prevent cardiovascular disease by inhibiting VEGF-dependent angiogenesis in atherosclerosis? *European Atherosclerosis Society 82nd Annual Congress.* May 2014

Rebecca L. Edwards, Paul A. Kroon. Can dietary polyphenols prevent cardiovascular disease by inhibiting VEGF-dependent angiogenesis in atherosclerosis? *John Innes Centre Annual Science Meeting.* October 2013

Can Dietary Polyphenols Reduce Atherosclerosis through Inhibition of VEGF Induced Angiogenesis? Rebecca Edwards, Ben Kirkup, Paul Kroon. *IFR student Science Showcase.* May 2013

Oral presentations

Edwards RL, Kroon PA. Pomegranate polyphenols and their metabolites interact with VEGF to inhibit VEGFR-2 phosphorylation. *Food Bioactives and Health Conference.* September 2016

Edwards RL, Kroon PA. Can polyphenols target angiogenesis in atherosclerosis? *IFR Coffee Break Science.* November 2015

Edwards RL, Kroon PA. Progress update on BACCHUS project WP5, Task 5.3.2. *4th European Union FP7 BACCHUS project meeting.* November 2015

Edwards RL, Kroon PA. Potent inhibition of VEGF activity by pomegranate polyphenols: A novel mechanism for the atheroprotective effects observed in epidemiology. *IFR student Science Showcase.* September 2015

Edwards RL, Kroon PA. Potent inhibition of VEGF activity by polyphenols: A novel mechanisms for the atheroprotective effects of polyphenols. *International Conference on Polyphenol and Health.* October 2015

Edwards RL, Kroon PA. Can dietary polyphenols prevent cardiovascular disease by inhibiting VEGF-dependent angiogenesis in atherosclerosis? *IFR Food and Health ISP Annual Meeting*. March 2014

Edwards RL, Kroon PA. Progress update on BACCHUS project WP5, Task 5.3.2. 3rd *European Union FP7 BACCHUS project meeting.* February 2014

Edwards RL, Kroon PA. Progress update on BACCHUS project WP5, Task 5.3.2. 2nd *European Union FP7 BACCHUS project meeting.* June 2013

Other

Edwards RL, Kroon PA. BACCHUS Month 48 project interim report update on progress towards deliverable 5.5. September 2016

Edwards RL, Kroon PA. BACCHUS Month 36 project interim report update on progress towards deliverable 5.5. September 2015

Edwards RL. Awarded best presentation prize at the *IFR student Science Showcase* for the talk titled: Potent inhibition of VEGF activity by pomegranate polyphenols: A novel mechanism for the atheroprotective effects observed in epidemiology. September 2015

Edwards RL, Kroon PA. BACCHUS Month 18 project interim report update on progress towards deliverable 5.5. March 2014

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Chapter 1

General Introduction

1.1 Summary of thesis aims

The data presented in this thesis surrounds the central investigation into the effects of dietary polyphenols on vascular endothelial growth factor (VEGF) induced VEGF receptor 2 (VEGFR-2) phosphorylation, and the impact this could have on pathological angiogenesis. The specific aims for the project were 1) to investigate if and how dietary polyphenols could inhibit VEGF induced VEGFR-2 phosphorylation in HUVECs, 2) to determine whether the polyphenols which elicit potent inhibition of VEGF-induced VEGFR-2 phosphorylation could, in turn, inhibit VEGF induced atherosclerosis. The purpose of this general introduction is to provide an overall understanding of the background to the project and will provide detailed information about polyphenols, VEGF and its associated signalling and the relevance of both of these to cardiovascular atherosclerotic disease.

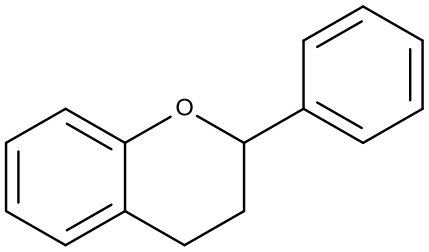
1.2 Classification of polyphenols

Polyphenols are a group of phytochemicals produced in a wide variety of plants, including fruits and vegetables. There is a large variety of polyphenols with ranging complexity each containing varying numbers of 6 carbon phenol ring structures and side groups (Cheynier, 2005). In general polyphenols can be classified as flavonoids, phenolic acids, lignans and stilbenes, although many polyphenols do not fall into any of these classifications (D'Archivio et al., 2007, Manach et al., 2004). An overview of the basic structure of the polyphenol classes can be seen in Figure 1.1. The predominant polyphenols consumed in the diet are flavonoids, although in high fibre, coffee or citrus diets this balance may shift (Clifford, 1999). Flavonoids can be further classified, for example into flavanols, flavonols, flavones, flavanones, isoflavones, and anthocyanins. A summary of the main flavonoid sub-classes can be seen in Figure 1.2.

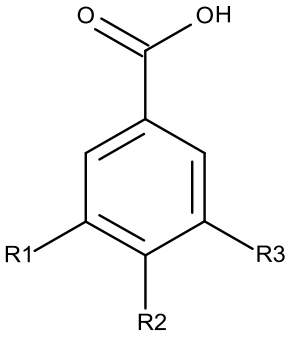
1.3 Polyphenol dietary intake

A number of studies have estimated the average intake of dietary polyphenols to determine the most widely consumed classes of polyphenols. Polyphenol intake varies depending on the country where the data is collected. This is due to the varying cultural preferences, for example within Japan isoflavone consumption is

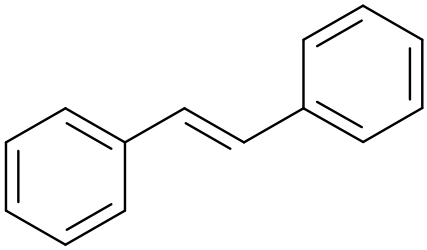
higher than in other countries due to the frequent consumption of soy products (Arai et al., 2000) while consumption of fruits may be higher in the diet of people from Mediterranean Europe versus non-Mediterranean Europe (Zamora-Ros et al., 2016, Murphy et al., 2014). Variation in total polyphenol consumption data also varies greatly due to limited data reported in some studies. For example, two reports from Finland focussed on flavonols/flavones (Hirvonen et al., 2000) and flavonoids (Knekt et al., 2002) intake found the daily dose of these compounds was 8 and 24.2 mg/day respectively. However, another study reported on the intake of a much larger range of polyphenols and estimated a daily total polyphenol dose of 863 mg/day (Ovaskainen et al., 2008). 75 % of the total polyphenol intake was phenolic acids which were not investigated in the 2 earlier reports. It has been identified that many global populations eat under 5 portions of fruit or vegetables per day which correlated with lower polyphenol consumption (Murphy et al., 2014), suggesting that daily polyphenol intake could increase with adherence to dietary recommendations. Globally, non-alcoholic beverages and specifically tea and coffee provide a large proportion of the daily polyphenol intake. In many countries ranging from Brazil (Miranda et al., 2016) to Finland (Ovaskainen et al., 2008) the phenolic acids, predominantly hydroxycinnamic acids, are the largest contributor to daily polyphenol intake. Where reported, the prevalent hydoxycinnamic acid in the diet was 5-caffeoylquinic acid (Miranda et al., 2016, Grosso et al., 2014) or its aglycone caffeic acid (Ovaskainen et al., 2008) with coffee being the main food source. In comparison to other European countries the UK has a higher intake of polyphenols from consumption of tea rather than coffee (Zamora-Ros et al., 2016), perhaps explaining the observation that flavanols are the predominant polyphenol in the UK diet (Yahya et al., 2016). When tea and coffee were taken out of consideration the hydroxycinnamic acids were once again the predominant class of polyphenol in the UK diet with potato, pears, tomato, carrot, “ready-to-cook” sauces, and soups identified as the sources (Yahya et al., 2016). For a summary of the data please see Table 1.1.



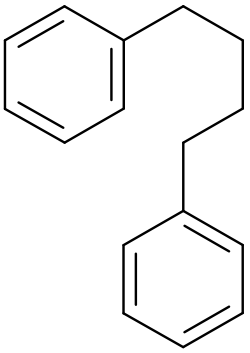
Flavonoid backbone



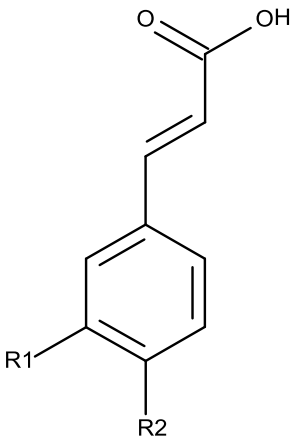
Hydroxybenzoic acid backbone



Stlibene backbone



Lignan backbone



Hydroxycinnamic acid backbone

Figure 1.1: The basic structures of the main polyphenol classes.

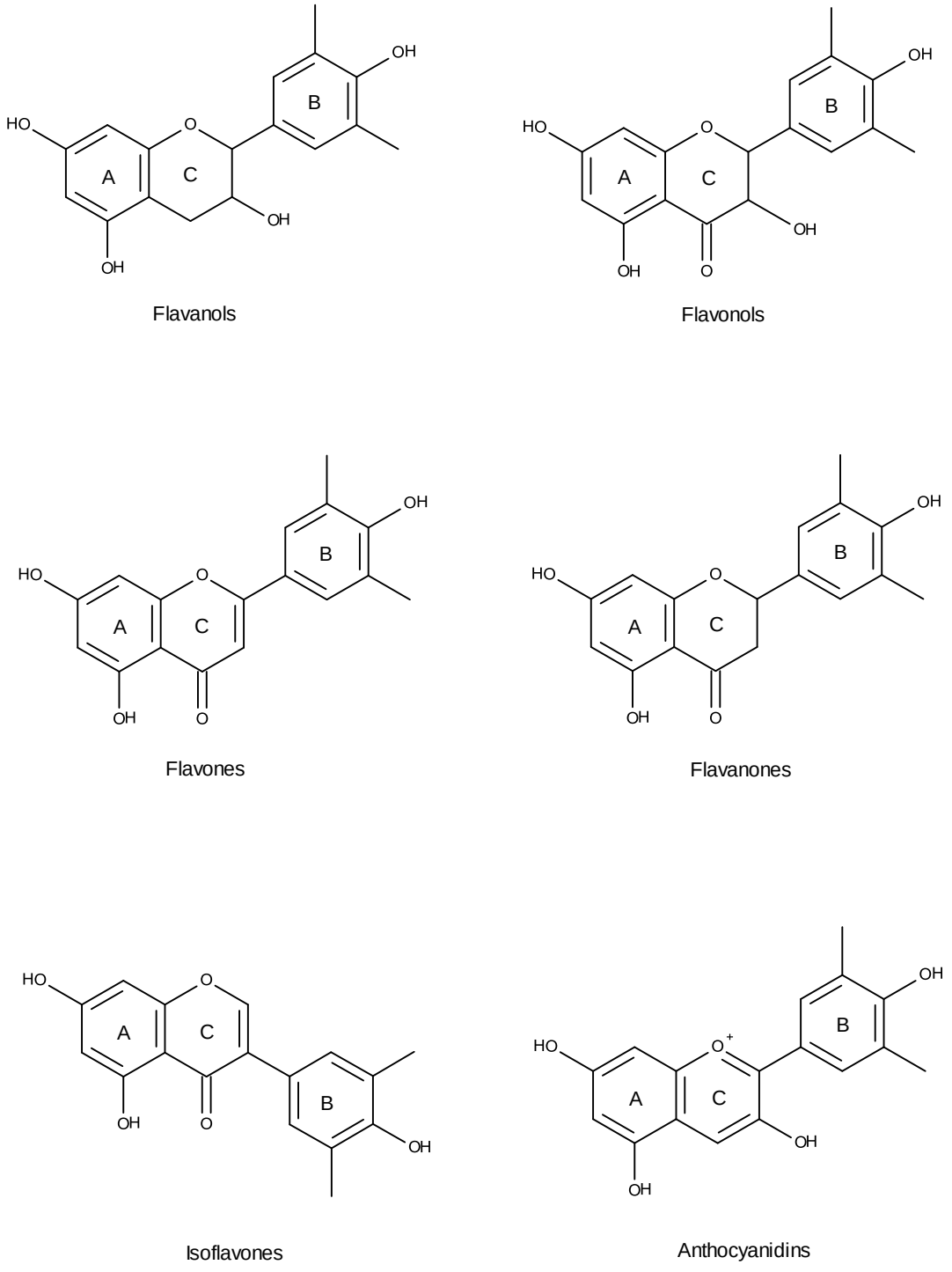


Figure 1.2: The main structures of the flavonoid subclasses

Table 1.1: Estimated daily polyphenol consumption (mg/day) and dietary sources of different countries or regions

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
Australia	454 mg/day	Anthocyanidins (2.6 mg/day)	Malvidin (1.56 mg/day)	Tea, citrus fruits, grapes	(Johannot and Somerset, 2006)
		Flavanols (422.5 mg/day)	Thearubigins (213.04 mg/day)		
		Flavanones (6.94 mg/day)	Hesperetin (4.75 mg/day)		
		Flavones (0.53 mg/day)	Apigenin (0.45 mg/day)		
		Flavonols (20.69 mg/day)	Quercetin (12.53 mg/day)		
Brazil	349 mg/day	Hydroxyinnamic acids (281.2 mg/day)	5-Caffeoylquinic acid (98.7 mg/day)	Coffee, citrus fruits	(Miranda et al., 2016)
		Hydroxybenzoic acids (3.4 mg/day)	Not specified		
		Flavanones (16.1 mg/day)	Not specified		
		Flavonols (14.6 mg/day)	Not specified		
		Flavanols (11.4 mg/day)	Not specified		
		Anthocyanins (6.8 mg/day)	Not specified		
		Flavones (3.6 mg/day)	Not specified		
		Isoflavonoids (1.5 mg/day)	Not specified		
		Lignans (2.3 mg/day)	Not specified		
		Other (8.1 mg/day)	4-Ethylguaiacol (0.8 mg/day)		
Europe	429 mg/day	Flavanols (245 mg/day)	Thearubigins (156 mg/day)	Non-alcoholic beverages, fruit	(Vogiatzoglou et al., 2015)
		Anthocyanidins (19 mg/day)	Cyanidin (9 mg/day)		
		Flavonols (23 md/day)	Quercetin (14 mg/day)		

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
		Flavanones (14 mg/day)	Hesperetin (9 mg/day)		
Europe cont.		Flavones (4 mg/day)	Apigenin (3 mg/day)		
		Proanthocyanidins (124 mg/day)	d.p. >10 (42 mg/day)		(Vogiatzoglou et al., 2015)
Finland	8 mg/day	Flavonols/flavones (8 mg/day)	Not specified	Not specified	(Hirvonen et al., 2000)
	24.2 mg/day	Flavonoids (24.2 mg/day)	Hesperetin (15.1 mg/day)	Citrus fruits, apples	(Knekt et al., 2002)
	863 mg/day	Phenolic acids (641 mg/day)	Caffeic acid (417 mg/day)	Coffee/tea, cereals, fruits	(Ovaskainen et al., 2008)
		Anthocyanidins (47 mg/day)	Cyanidin (23 mg/day)		
		Flavanols (5.4 mg/day)	Not specified		
		Flavanones (27 mg/day)	Not specified		
		Isoflavanones (0.9 mg/day)	Not specified		
		Proanthocyanidins (128 mg/day)	d.p. >10 (21 mg/day)		
		Ellagitannins (12 mg/day)	Not specified		
Greece	161 mg/day	Flavones (7 mg/day)	Apigenin (5 mg/day)	Grapes, wine and peaches	(Dilis and Trichopoulou, 2010)
		Flavonols (28 mg/day)	Quercetin (18 mg/day)		
		Flavanones (27 mg/day)	Hesperetin (19 mg/day)		
		Flavan-3-ols (14 mg/day)	Catechin/epicatechin (6 mg/day)		
		Anthocyanidins (10 mg/day)	Cyanidin/delphinidin (4 mg/day)		
		Isoflavonoids (<0.1 mg/day)	Not specified		

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
		Proanthocyanidins (75 mg/day)	Not specified		
Italy	251 mg/day	Proanthocyanidins (96.1 mg/day)	Not specified	Fruit, red wine	(Ponzo et al., 2015)
		Flavanols (50.4 mg/day)			
Italy cont.		Anthocyanidins (32.9 mg/day)			
		Flavanones (24.2 mg/day)			(Ponzo et al., 2015)
		Flavonols (14.4 mg/day)			
		Flavones (1.2 mg/day)			
		Isoflavonoids (0.7 mg/day)			
	348.8	Flavonols (17 mg/day)	Not specified	Seasonal fruits, citrus fruits and leafy vegetables	(Pounis et al., 2016)
		Flavones (0.7 mg/day)			
		Flavanones (32.4 mg/day)			
		Flavanols (51.2 mg/day)			
		Anthocyanidins (144 mg/day)			
		Isoflavonoids (23.5 mg/day)			
		Lignans (80 mg/day)			
Japan	63.9 mg/day	Isoflavonoids (47.2 mg/day)	Genistein (30.5 mg/day)	Tofu, natto, miso, onions	(Arai et al., 2000)
		Flavonoids (16.7 mg/day)	Quercetin (9.3 mg/day)		
	1492 mg/day	Total polyphenols	Not specified	Coffee, green tea	(Taguchi et al., 2015)
Poland	1740.7 mg/day	Flavanols (637.3 mg/day)	Gallocatechin (72.6 mg/day)	Tea, coffee, chocolate	(Grosso et al., 2014)
		Flavonols (106.1 mg/day)	Not specified		

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
		Flavanones (103.8 mg/day)	Not specified		
		Flavones (15.5 mg/day)	Not specified		
		Anthocyanins (29.7 mg/day)	Hesperidin (28.1 mg/day)		
Poland cont.		Isoflavonoids (1.6 mg/day)	Not specified		
		Hydroxybenzoic acids (94.6 mg/day)	5-O-galloylquinic acid (60.8 mg/day)		(Grosso et al., 2014)
		Hydroxycinnamic acids (705.5 mg/day)	5-caffeoylquinic acid (224.6 mg/day)		
		Lignans (0.6 mg/day)	Not specified		
		Stilbenes (0.2 mg/day)	Not specified		
		Other (41.5 mg/day)	Not specified		
The Netherlands	25.9 mg/day	Flavonoid (25.9 mg/day)	Quercetin (16.3 mg/day)	Tea, onions, apples	(Hertog et al., 1993)
	50 mg/day	Flavonoids (50 mg/day)	Epicatechin gallate (16.5 mg/day)	Tea, chocolate, apples/pears	(Arts et al., 2001a)
	< 1 mg/day	Isoflavonoids (0.88 mg/day)	Daidzein (0.15 mg/day)	Peas/beans, cereal, bread	(Boker et al., 2002)
Spain	820 mg/day	Phenolic acids (302.7 mg/day)	Not specified	Coffee, oranges, red wine, apples	(Tresserra-Rimbau et al., 2012)
		Flavanones (132 mg/day)			
		Proanthocyanidins (117 mg/day)			
		Flavonols (80.4 mg/day)			
		Flavones (41.6 mg/day)			

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
		Anthocyanins (38.5 mg/day)			
		Flavanols (27.0 mg/day)			
		Other (79.7 mg/day)			
UK	1292 mg/day	Flavanols (588.9 mg/day)	Not specified	Tea, coffee, onion, potato, tomato	(Yahya et al., 2016)
UK cont.		Flavonols (60.7 mg/day)			
		Flavanones (24.3 mg/day)			
		Anthocyanins (19.9 mg/day)			
		Flavones (3.1 mg/day)			
		Isoflavonoids (3.2 mg/day)			
		Phenolic acids (545 mg/day)			
		Other (48.1 mg/day)			
	808 mg/day	Flavanols (391.5 mg/day)	Not specified	Tea, coffee, onion, potato, tomato	(Yahya et al., 2016)
		Flavonols (55.2 mg/day)			
		Flavanones (16.8 mg/day)			
		Anthocyanins (18.6 mg/day)			
		Flavones (2.4 mg/day)			
		Isoflavonoids (1.2 mg/day)			
		Phenolic acids (282 mg/day)			
		Other (40.5 mg/day)			
USA	189.7 mg/day	Flavanols (158.4 mg/day)	Not specified	Tea	(Chun et al., 2007)

Country	Daily polyphenol intake	Polyphenols analysed	Main polyphenol per class	Key food sources	Ref.
		Flavanones (14.4 mg/day)			
		Flavonols (12.9 mg/day)			
		Anthocyanidins (3 mg/day)			
		Flavones (1.5 mg/day)			(Chun et al., 2007)
USA cont.	207 mg/day	Flavonoids (207 mg/day)	Not specified	Tea	(Chun et al., 2010)
	< 1 mg/day	Isoflavonoids (0.15 mg/day)	Genistein (0.07 mg/day)	Fruit, grains, berries	(de Kleijn et al., 2001)
		Lignans (0.58 mg/day)	Secoisolariciresinol (0.56 mg/day)		
	57.7 mg/day	Proanthocyanidin	d.p. >10 (21 mg/day)	Apples, chocolate and grapes	(Gu et al., 2004)
	95 mg/day	Proanthocyanidin	d.p. >10 (28.5 mg/day)	Tea, legumes, and wines	(Wang et al., 2011)

1.4 Polyphenol metabolism

Polyphenol metabolism can occur through alterations, for example hydrolysis and oxidation or conjugation of a side group to the molecule, for example a glucuronide or sulfate group. Polyphenol metabolism primarily occurs in the small intestine, although further conjugation occurs within the liver (Liu and Hu, 2002, Donovan et al., 2001, Crespy et al., 2001, Spencer et al., 1999) and it is thought the majority of polyphenols are likely to first undergo microbial alterations before being conjugated by the liver (Rechner et al., 2002). Hydrolysis of flavonoid glycosides occurs in the upper intestine (Liu and Hu, 2002) with the intestinal epithelial hydrolysing enzymes playing a key role (Németh et al., 2003).

1.4.1 Green tea polyphenol metabolism

The green tea catechins are a group of flavan-3-ols found in high quantities within green tea. The majority of these green tea catechins are gallated while the predominant polyphenol in a wide range of green tea cultivars is epigallocatechin gallate (EGCG) (Lee et al., 2014, Kerio et al., 2013). The structures of the green tea catechins can be seen in Figure 1.3. Epicatechin (EC) has been shown to undergo conjugation to a sulfate group via the actions of sulfotransferases SULT1A1 and SULT1A3 (Vaidyanathan and Walle, 2002). Liver isolated catechol o-methyltransferase (COMT) was found to methylate both EGCG and epigallocatechin (EGC) although rat derived COMT was more efficient at this action (Lu et al., 2003). Rat, but not human, liver fraction efficiently glucuronidated epicatechin (Vaidyanathan and Walle, 2002) although it has been suggested that in rats the majority of glucuronidation of epicatechin occurs within the intestinal mucosa (Piskula and Terao, 1998). Similarly, in mice the majority of EGCG was identified as a glucuronide conjugate following pure compound ingestion (Lambert et al., 2003). In humans EC appears to be predominantly in the glucuronide conjugated form, although sulfates and methyl sulfates have been observed in plasma following consumption of high EC containing foods (Actis-Goretta et al., 2012). This being said, two isoforms of uridine 5'-diphospho-glucuronosyltransferase (UGT) found in humans were unable to glucuronidate EC *in vitro* (Vaidyanathan and Walle, 2002). However, it is important to note that while EC is commonly found in the plasma in a

conjugated form, epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) have been found unconjugated in plasma (Stalmach et al., 2009, Chow et al., 2001, Ullmann et al., 2003).

It has also been demonstrated that EGCG can be hydrolysed by common gastrointestinal bacteria, e.g. *Enterobacter aerogenes*, to form EGC and gallic acid (Takagaki and Nanjo, 2010). Both *in vivo* feeding of EGCG in rats and *in vitro* incubation of rat cecal bacteria with EGC established the predominant terminal metabolite as 5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid, although reasonable amounts of 5-(3,5-dihydroxyphenyl)-γ-valerolactone was also detected (Takagaki and Nanjo, 2010). Cleavage of EGCG to EGC and gallic acid, as well as the conversion of EGC to 3', 4', 5'-trihydroxyphenyl-γ-valerolactone was observed following incubation of the green tea catechins with ileostomy fluids (Schantz et al., 2010).

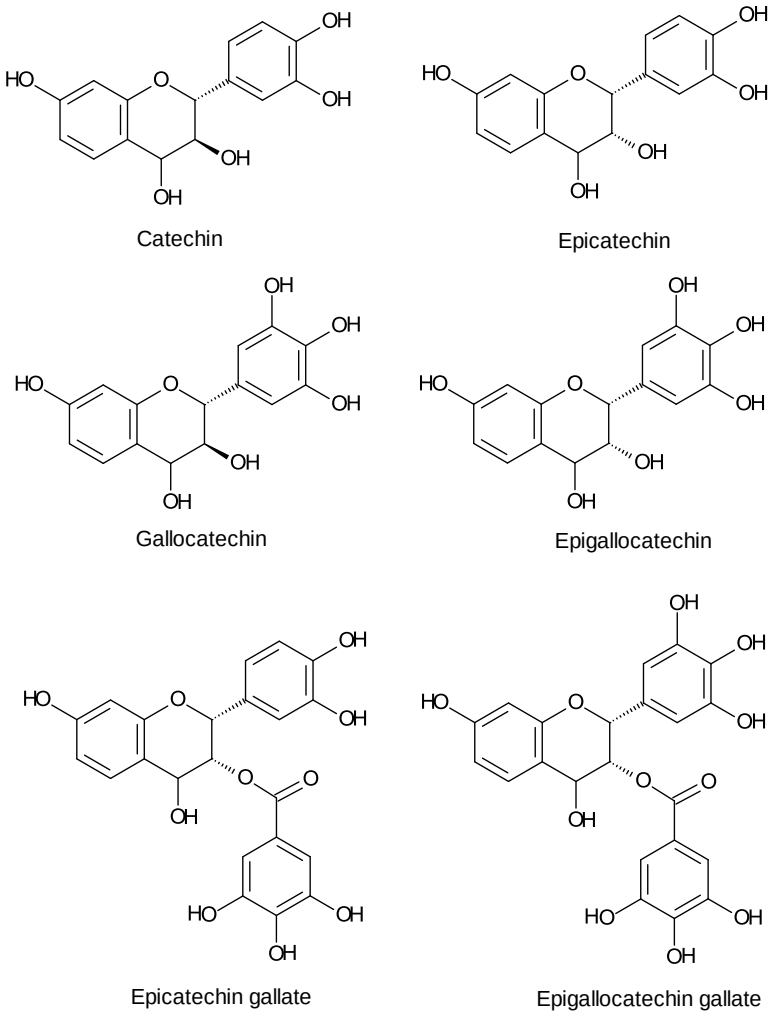


Figure 1.3: The chemical structures of green tea catechins.

1.4.2 Pomegranate polyphenol metabolism

Ellagitannins are converted to ellagic acid, first by hydrolysis to form hexahydroxydiphenic acid which then undergoes spontaneous lactone formation to form ellagic acid (Larrosa et al., 2006) (see Figure 1.4 for diagram). Incubation of an ellagitannin with cecal content at the pH found within the small intestine caused significant release of ellagic acid (Daniel et al., 1991) while 241 % of ellagic acid was recovered from the ileostomy fluid following raspberry consumption (Gonzalez-Barrio et al., 2010) demonstrating the rapid rate of conversion early within the gastrointestinal system. This was confirmed in the Iberian pig model (Espín et al., 2007). Given the high microbial content of the cecum it was suggested that microbes may be performing this action (Daniel et al., 1991) through enzymatic hydrolysis (Huang et al., 2007). A number of enzymes have been implicated in this action including ellagitannin acyl hydrolase, cellulase, xylanase, and β glucosidase (Xu et al., 2014a, Vattem and Shetty, 2003, Huang et al., 2008, Huang et al., 2007). Bacteria, particularly *Lactobacilli sp.*, are implicated in the ability to produce tannin acyl hydrolases to undertake this hydrolysis action (Vaquero et al., 2004, Osawa et al., 2000). It should be noted however that an earlier report found β-glucosidase, esterases, and α-amylase did not have an effect on ellagic acid release from a raspberry extract, although in this experiment components of the raspberry extract inhibited the enzyme activity (Daniel et al., 1991).

Once ellagic acid has formed it undergoes further hydrolysis by the microbiota within the jejunum region of the gastrointestinal tract to produce the urolithins (Espín et al., 2007). First the intermediary urolithin, the pentahydroxy-uroltihin M5 is produced after which the most widely discussed sequential hydrolysis of the urolithins proceeds as follows: tetrahydroxy-urolithin D, trihydroxy-urolithin C, dihydroxy-urolithin A or isourolithin A, and finally monohydroxy-urolithin B. It should be noted that 2 other tetrahydroxy-urolithins (urolithin M6 and urolithin E) and 1 other trihydroxy-urolithin (urolithin M7) have been recently identified but are thought to be rapidly converted into urolithin A (García-Villalba et al., 2013). See Figure 1.5 for a diagram depicting the current understanding about microbial conversion of ellagic acid to the urolithins. The predominant urolithin found in

circulation is urolithin A. Urolithin B tends to form from isourolithin A rather than urolithin A and this has led to the hypothesis that there are three distinct urolithin phenotypes based on the terminal urolithins produced by each individual (Tomás-Barberán et al., 2014).

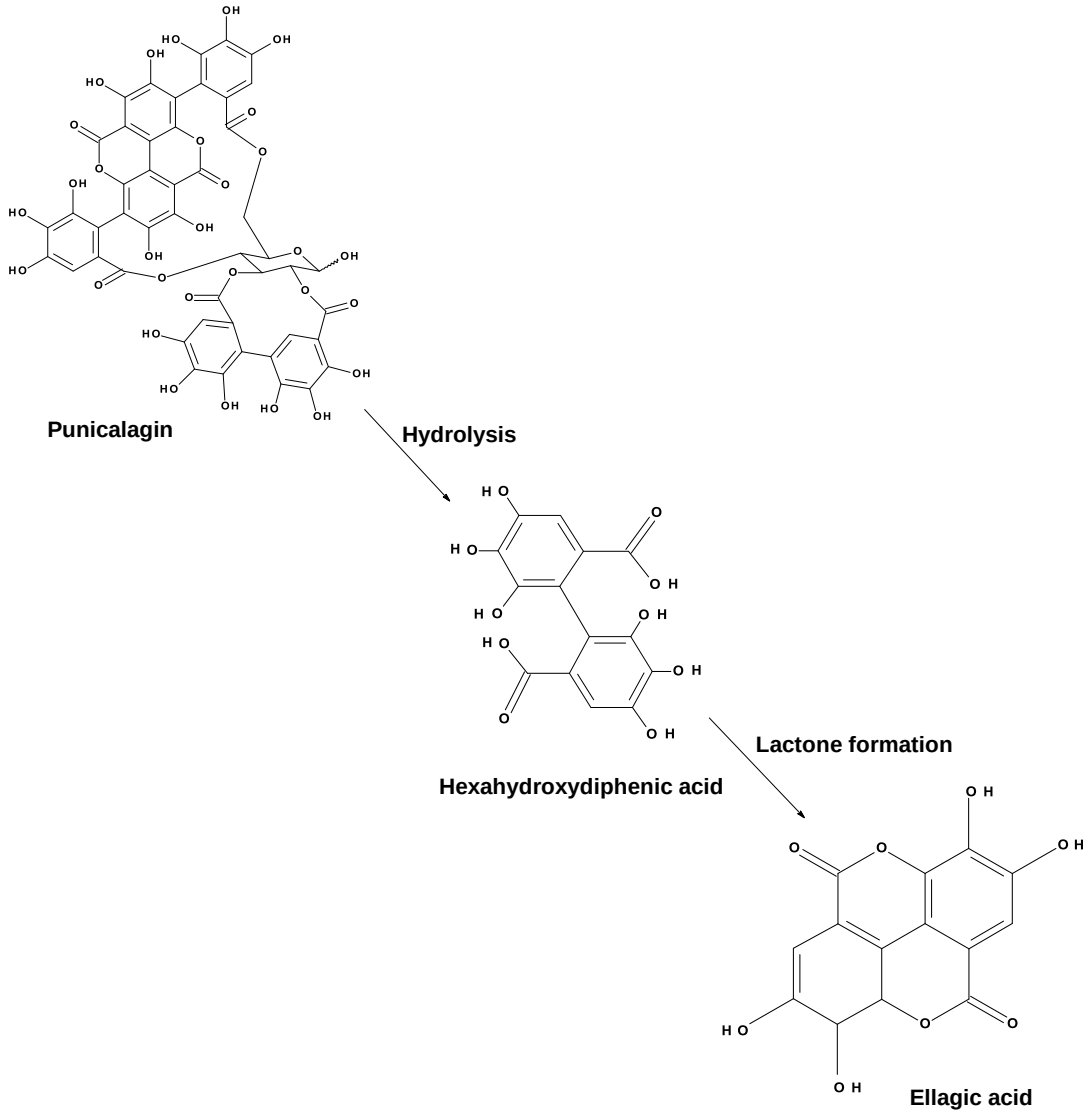


Figure 1.4: The conversion of punicalagin to ellagic acid

"Phenotype A" produce only urolithin A metabolites, "phenotype B" produce isourolithin A/urolithin B and urolithin A metabolites, and "phenotype 0" none of these metabolites. While the research into the bacterial species responsible for the production of urolithins is still in its infancy it has been suggested that *firmicutes* species play a role in the metabolism of ellagic acid to the urolithins (García-Villalba et al., 2013). Furthermore, urolithin A correlated to the abundance of *Gonidobacter*

in the faeces while *Lactobacillus*, *Leuconostoc* and *Pediococcus* are tentatively linked to isourolithin A/urolithin B production. The production of ellagic acid and tetrahydroxy-urolithins is tentatively linked to *bacteroidetes* (Romo-Vaquero et al., 2015).

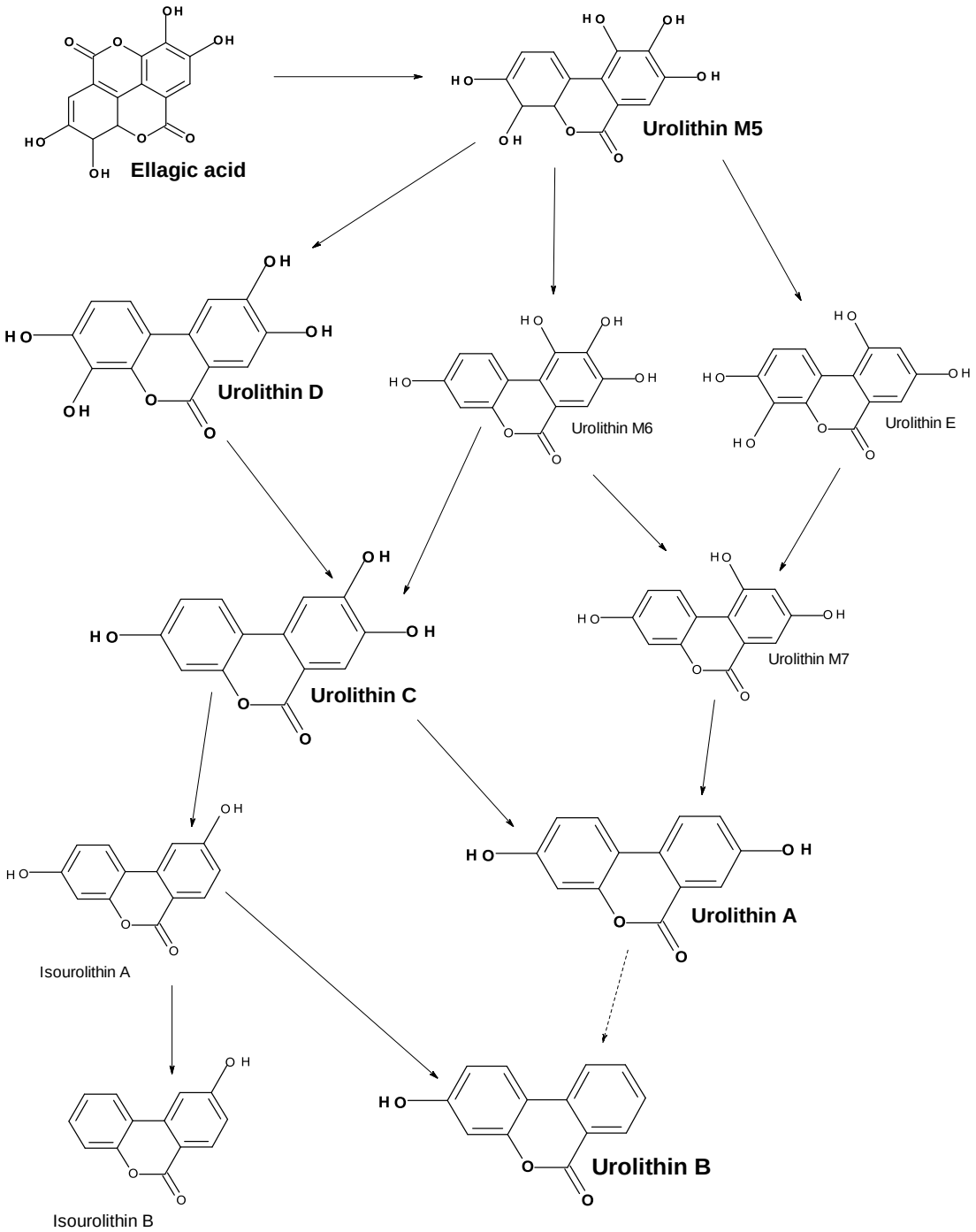


Figure 1.5: The route of microbial metabolite production from ellagic acid.

1.5 Bioavailability of polyphenols

In 2005 Manach et al. (Manach et al., 2005) undertook a comprehensive review of 97 bioavailability studies focussed on 18 individual polyphenols commonly found in the diet. This review provides an excellent overview of the bioavailability of these 18 compounds, allowing comparison by normalising the bioavailability to a 50 mg aglycone dose. Gallic acid and the isoflavones proved most bioavailable with concentrations of up to 4 µM reported in the plasma from a 50 mg dose. The sugar moiety attached to flavonoids can significantly alter the bioavailability, for example rutin had a much lower C_{max} than the quercetin glucoside, most likely due to the requirement for cleavage of the rutinoside further down the digestive tract in the colon (Hollman et al., 1999). Galloylation of epigallocatechin also significantly impacted the bioavailability of the polyphenol reducing the achievable C_{max} from a 50 mg dose from 1.1 µM to 0.12 µM. The least bioavailable compounds identified in the review by Manach et al. were the anthocyanins (C_{max} 0.03 µM) and the proanthocyanadin dimers (C_{max} 0.02 µM).

1.5.1 Bioavailability of green tea polyphenols

Studies investigating the bioavailability of the predominant green tea catechin, EGCG, have reported quite large differences in bioavailability although it has been suggested that earlier reports have underestimated due to the lack of consideration for the methylated conjugates (Meng et al., 2002). Following ingestion of a 1600 mg dose of pure EGCG a peak plasma concentration of 7 µM was found in participants (Ullmann et al., 2003) while others reported plasma concentrations in the nanomolar range from lower doses. Henning et al. found that following consumption of tea containing 193 mg green tea catechins produced an EGCG plasma concentration of 174 nM. They also reported that EGCG was more bioavailable when given as a green tea supplement instead of tea in the same pool of participants, resulting in 326 nM peak EGCG plasma concentrations (Henning et al., 2004). It is also interesting to note that the EGCG compounds identified in the plasma have been found up to 90 % in the aglycone form (Ullmann et al., 2003, Chow et al., 2001, Meng et al., 2002, Lee et al., 2002). Bioavailability studies in animals found that gastric delivery of polyphenols at varying doses produced peak

plasma concentration of around 31-50 nM (Lambert et al., 2003, Dube et al., 2011a, Lambert et al., 2004). However, it has also been shown that green tea catechins can be rapidly glucuronidated after entry into the plasma and are found in large quantities as phase-2 conjugates (Lambert et al., 2003). All studies demonstrate that green tea catechins are highly unstable and are rapidly turned over in the body.

1.5.2 Bioavailability of pomegranate polyphenols

Reported studies of the bioavailability of ellagitannins and ellagic acid in humans to date have mainly focussed from pomegranate food products, while some reports use extracts and, at the time of writing, only one study investigated the bioavailability of pure ellagic acid. No human bioavailability trial found punicalagin, the pomegranate derived ellagitannin, in the plasma of volunteers. It should be noted that punicalagin was found at 30 µg/ml in the plasma of rats following feeding with a punicalagin supplemented diet but has not been observed in the plasma from any other interventions since this trial (Cerda et al., 2003). Ellagic acid also has relatively poor bioavailability, with only nanomolar concentrations of ellagic acid observed in human plasma of (20-100 nM) following consumption of pomegranate products such as juice or extracts (Seeram et al., 2004, Seeram et al., 2006, Mertens-Talcott et al., 2006). In a study where participants were given a dose of pure ellagic acid (40 mg) a higher plasma concentration of 660 nM was reported (Hamad et al., 2009). Studies in rats report peak plasma ellagic acid concentrations of just over 200 nM following dietary intervention with either pure ellagic acid or pomegranate extract (Murugan et al., 2009, Lei et al., 2003) although concentrations of up to 16.5 µM have been reported (Cerda et al., 2003). Studies on ellagic acid bioavailability in mice either from pomegranate products or pure compounds are limited. An early study in 1986 found little to no absorption of ellagic acid when incorporated into the diet at a 1 % concentration (Smart et al., 1986). A later study reported a mean peak plasma concentration of 38 nM when a dose of 0.8 mg pomegranate extract was given (Seeram et al., 2007), but research remains limited.

More recently the microbial metabolites of ellagic acid, the urolithins (Cerda et al., 2005), have been suggested as the bioactive compounds associated with the health benefits of pomegranate products due to the their increased bioavailability over the parent compounds (Cerda et al., 2004, González-Sarrías et al., 2015). While a higher ellagic acid dose did not increase the plasma concentration of ellagic acid it did increase the production of the microbial metabolites, the urolithins (González-Sarrías et al., 2015). The urolithins differ from one another in the number of hydroxyl groups contained in the structure and their position. The Iberian pig model has been used extensively to clarify the microbial breakdown of ellagic acid (Espín et al., 2007). The microbiota of the small intestine converted ellagic acid into the first urolithins however the terminal urolithins, particularly urolithin B, were only produced in the large intestine. The requirement of colonic microflora for the production of urolithin A and B glucuronides was confirmed in ileostomy patients where these compounds were not found in the illeal fluid (Gonzalez-Barrio et al., 2010). It is suggested that the microbiota composition may play a role in the ability of an individual to gain benefit from ellagitannin consumption (Puupponen-Pimiä et al., 2013). The uptake of urolithin A, B and C into the intestine tissue was reasonable while only small amounts of urolithin D was able to pass into the tissue. Quantification of the urolithins in human plasma has shown concentrations exceeding 18 µM (Cerda et al., 2004) with urolithin A and B being reported at the highest concentrations.

1.6 Pathology of atherosclerosis

Atherosclerosis is a disease which has been observed across the world for thousands of years (Thompson et al., 2013). The world health organisation (WHO) reports that the biggest cause of death globally is cardiovascular disease, and primarily ischemic heart disease. In western countries the prevalence of atherosclerosis has been linked to age as well as more modern factors such as smoking status and obesity (Robinson et al., 2009, Webber et al., 2012).

The risk of a person developing cardiovascular disease is commonly measured using the Framingham cardiac risk scale (D'Agostino et al., 2008). This scale considers a number of contributing factors: diabetes mellitus (or other equivalent disease),

smoking status, age, gender, total cholesterol levels, HDL cholesterol levels, systolic blood pressure and systolic blood pressure after treatment (if applicable). The pattern of progression differs between genders with the risk of atherosclerotic development in men increasing linearly over time whereas for women the risk significantly began to increase from around the age of 50 (Joakimsen et al., 1999). These age-related differences observed between the sexes are however lost within the very elderly (Sawabe et al., 2006).

Atherosclerosis is characterised as a thickening of the arterial wall through deposits of lipids, LDL cholesterol, smooth muscle cells, foamy macrophages and platelets. Atherosclerosis is initiated in adolescence (Strong et al., 1999) but these early lesions are thought to not affect lumen size or display any physiological symptoms of plaque development and that the lesions can regress (Nissen, 2000). Progression of atherosclerosis into an unstable and continuous stage of the disease typically occurs beyond the age of 50 [147] although it can occur earlier in certain individuals, for example those with a genetic disorder such as familial hypercholesterolemia (Lavrencic et al., 1996). Atherosclerosis typically starts at regions of differential blood flow rates which are known to alter sheer stress and the duration circulating components known to advance atherosclerosis are in these regions of the circulatory system, repeatedly demonstrating an ability to gather at these regions more readily (Sims, 1985, Ku et al., 1985, Zarins et al., 1983). Atherosclerosis begins with an increase in oxidised LDL within the tissue which typically accumulates because of high LDL concentration within the blood. It has been shown that even in clinically healthy patients that a higher LDL cholesterol level is correlated to thicker vessel intima [151]. This build-up results in the release of chemokines and monocyte adhesion molecules which attract immune cells to the site [148]. These adhesion molecules, such as P-selectin, adhere to the monocytes increasing the movement of these cells into the tissue [149]. Once in the intima the monocytes mature into macrophages and take up the oxidised LDL through phagocytosis forming foam cells. Small collections of foam cells and oxidised LDL are known as fatty streaks and can be taken as an early indication of future atherosclerosis but, due to the occurrence of regression, is not a diagnosis for the

disease itself. Following fatty streak formation the intima of the vessel wall begins to thicken, encroaching on the lumen space. Lipids gather in pools within the layers of smooth muscle cells and this gathering is associated with increased macrophage infiltration and increased apoptosis. A reduction in extracellular matrix proteins also occurs and a necrotic core forms (Otsuka et al., 2015). Following lipid pool expansion and necrotic core development a fibrous cap across the lesion begins to form from fibrous smooth muscle cells with a high collagen content, although the mechanism is still poorly understood (Newby and Zaltsman, 1999). These are subject to proteolysis, for example from matrix metalloproteinases released from macrophages (Shah et al., 1995, Peeters et al., 2011), leaving the cap weakened increasing the risk of plaque rupture leaving the LDL cholesterol and foam cell enlarged intima exposed, typically forming a thrombus within the vessel. Furthermore, as the plaque grows it becomes increasingly vascularised (Hiyama et al., 2010). This vascularisation overcomes the hypoxia induced through plaque growth (Bjornheden et al., 1999). This increase in vascularisation is also linked to the instability of plaques which can lead to plaque rupture and haemorrhage (Fleiner et al., 2004, Kolodgie et al., 2007). It is thought that the majority of myocardial infarction actually stems from plaque rupture thrombus formation instead of total lumen occlusion by the plaque itself [154]. Repeated plaque rupture leads to a build-up of fibrous tissue increasing the plaque size [151]. A diagrammatic representation of atherosclerosis can be seen in Figure 1.6 while the morphological features of the atherosclerotic plaque from the study discussed in chapter 3 of this thesis can be seen in Figure 3.2.

To undertake studies investigating the impact of treatments on atherosclerosis a suitable model with a similar plaque progression is required. There are numerous cholesterol driven animal models of atherosclerosis such as the LDL receptor knockout (LDLR-/-) mouse or high cholesterol diet in other rodents. However, the most widely used model is the Apolipoprotein E knockout mouse (ApoE-/-) which has reduced clearance of very low density lipoproteins (vLDLs) and intermediary density lipoproteins (IDLs) (Meir and Leitersdorf, 2004, Plump et al., 1992). These mice develop spontaneous atherosclerosis even on a standard chow diet with the

plaque morphology mimicking that of atherosclerotic lesions in humans and other species resulting in it being the leading model for investigations into atherosclerosis (Reddick et al., 1994, Nakashima et al., 1994).

1.7 Impact of dietary polyphenols on CVD

1.7.1 Epidemiological and dietary intervention studies

The correlation between polyphenols or foods rich in polyphenols and a reduced risk in cardiovascular disease (CVD) associated factors has been understood for a while. Tea and green tea which are rich in polyphenols including flavan-3-ols have frequently been associated with a reduction in risk of myocardial infarction (MI) and stroke (Pang et al., 2016, Sesso et al., 2003) as well as a reduction in atherosclerosis (Sasazuki et al., 2000, Geleijnse Jm, 1999). Olive oil and the Mediterranean diet have also been associated with positive changes in blood pressure, lipid profiles and reductions in intima-media thickness (Martinez-Gonzalez et al., 2015, Hernaez et al., 2014, Fito et al., 2008). Daily pomegranate juice consumption, known to be a rich source of ellagitannins and anthocyanins (Gil et al., 2000), was able to reduce stress-induced cardiac ischemia (Sumner et al., 2005). While these studies demonstrate a strong link between foods containing large amounts of polyphenols and reduced CVD risk, the studies did not go further into which compounds were responsible for the effects. However, other studies have highlighted the link between certain polyphenols or classes of polyphenols which are also associated with reduced risk or occurrence of CVD. A higher consumption of flavonoids from a variety of sources has been linked to reduced mortality from cardiovascular events including ischemia and improvement in plasma lipid parameters (Yochum et al., 1999, Geleijnse et al., 2002, Gorinstein et al., 2006, Widmer et al., 2013). The Zupthen elderly study conducted in The Netherlands collated information about the diets of over 800 elderly men (aged 65-84) along with both CVD mortality data after 5 and 10 years follow up. Both reports found a higher flavonoid intake was inversely correlated to general CVD related mortality (Hertog et al., 1993) while after 10 years the risk of death specifically from myocardial infarction was also reduced in those with a higher flavonoid-containing diet (Hertog et al., 1997). In the same cohort an increased consumption of catechin

was associated with a reduction in ischemic heart disease (Arts et al., 2001b). Association of the flavonoid subclasses with cardiovascular improvements have also been investigated. In a Finnish cohort the flavanones and flavones were associated with reduced risk of CVD mortality while in the same cohort the flavonols and flavan-3-ols were associated with reduced incidence of stroke (Mursu et al., 2008). In a larger cohort an increased diet content of quercetin, kaempferol, naringenin and hesperetin was associated with reduced ischemia and stroke (Knekt et al., 2002). A summary of studies reporting an association between polyphenols and CVD risk and events can be seen in Table 1.2.Atherosclerosis research samples can be difficult or inappropriate to obtain as these require invasive procedures. Critical insights into atherosclerosis have come from gross evaluation of arterial plaque in post-mortem samples (Webber et al., 2012). Imaging techniques can provide clues to the degree of vessel wall thickening (measured as intima media thickness) and vessel stiffness (measured as flow mediated dilatation). However, to evaluate plaque progression under experimental conditions animal models are useful.

1.7.2 Polyphenol intervention trials in animals

Investigations into the anti-atherosclerotic effects of polyphenols have predominantly been undertaken with flavonoids although the stilbene resveratrol has also been investigated. Naringin and naringenin reduced atherosclerotic plaque size in both New Zealand white rabbits and wild type mice fed a high cholesterol diet (Lee et al., 2001, Chanet et al., 2012) however naringin diet supplementation did not reduce atherosclerotic plaque size in ApoE-/- mice (Chanet et al., 2012). Catechin, quercetin, and resveratrol reduced atherosclerotic plaque size by around 80 % in high cholesterol fed Syrian golden hamsters (Auger et al., 2005). The LDLR-/- mouse is also an important model of atherosclerosis and quercetin was reported to have potent anti-atherosclerotic effects in this model even after only a short 4 week intervention (Leckey et al., 2010). Quercetin also demonstrated anti-atherosclerotic effect in the high cholesterol fed New Zealand white rabbit model (Juzwiak et al., 2005). Ellagic acid limited atherosclerosis progression in high fat diet fed wild type mice but not in the ApoE-/- mouse model due to low plaque size in all groups (Ding et al., 2014). Arguably the most widely used animal model of atherosclerosis is the

ApoE-/- mouse model, and a large amount of the research investigating the anti-atherosclerotic effects of polyphenols has been undertaken in this model. Polyphenol rich food extracts, from products such as green tea, pomegranate, and apple peel rich in the polyphenols EGCG, punicalagin, and quercetin have all demonstrated strong inhibition of atherosclerosis growth after dietary intervention over the experimental period in ApoE-/- mice (Miura et al., 2001, Aviram et al., 2008, Gonzalez et al., 2015). Other alternative polyphenol rich extracts, such as pine bark extract rich in proanthocyanidins have also limited the size of the atherosclerotic plaque in the aortic sinus, the region of differential pressure around the semi-lunar valves of the aorta near the aortic root, in comparison to controls (Sato et al., 2009). A summary of polyphenols rich extracts or products and their effects on atherosclerosis plaque size in the ApoE-/- mouse model can be seen in Table 1.3. Specific polyphenols have also been investigated for their anti-atherogenic properties in the ApoE-/- mouse model. EGCG treatment, either in the drinking water or through intraperitoneal injection, reduced atherosclerotic plaque size in diet-induced accelerated atherosclerosis (Chyu et al., 2004, Yin et al., 2016). A mixture of catechin, caffeic acid and resveratrol was also able to significantly reduce western-diet accelerated atherosclerosis within the aortic sinus and the ascending aorta by up to 40 % (Norata et al., 2007). In this study we cannot conclude that any individual polyphenol may be responsible for the observed effect however both catechin and caffeic acid phenethyl ester have been independently reported to limit atherosclerosis progression in the ApoE-/- mouse (Hishikawa et al., 2005, Auclair et al., 2009). While diet supplementation with 0.01 % resveratrol was able to inhibit atherosclerosis progression by 52 %, in the ApoE*3, a more atherosclerosis susceptible ApoE genotype (Berbee et al., 2013), resveratrol has yet to be independently tested in the ApoE-/- model. Long term diet supplementation of quercetin (Shen et al., 2013) theaflavin (Loke et al., 2010), and the anthocyanin metabolite protocatechuic acid (Wang et al., 2010) were also able to inhibit atherosclerosis progression. A summary of the anti-atherosclerotic effects of individual polyphenols can be found in Table 1.4.

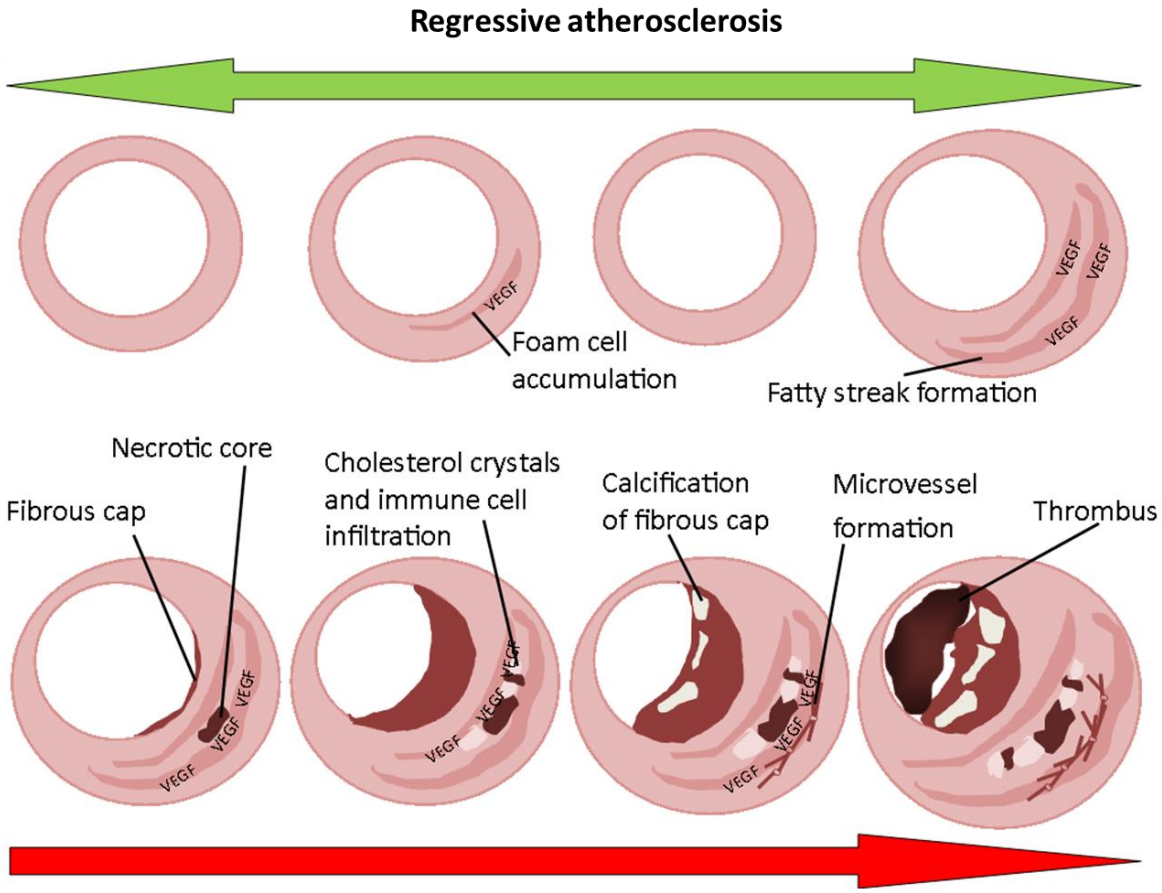


Figure 1.6: The morphological features of atherosclerotic disease

Table 1.2: Summary of the associations between polyphenols in the diet and reduced cardiovascular risk or events

Polyphenol Source	Polyphenol	Dose	Study population	No. participants	Outcome at follow up	Ref.
General diet	Flavonoid	25.9 m/day	Elderly men from The Netherlands	805	Reduced mortality from CVD	(Hertog et al., 1993)
General diet	Flavonoid	> 29.9 mg/day	Elderly men from The Netherlands	804	Reduced mortality from CVD and reduced MI	(Hertog et al., 1997)
General diet	Flavonoid	13.9 mg/day	Post-menopausal American women	34,492	Reduced risk of CHD mortality	(Yochum et al., 1999)
Tea	Flavonoid	32.9 mg/day	Men and Women from The Netherlands ≥55 years old	4807	Reduced risk of MI	(Geleijnse et al., 2002)
General diet	Flavonols	3.8 mg/day	Greek cohort ≥ 49 years old with peripheral arterial occlusive disease	200	Reduced risk of further peripheral arterial occlusive disease	(Lagiou et al., 2006)
	Flavones	0.8 mg/day				
	Flavan-3-ols	7.2 mg/day				
General diet	Flavanone, flavone	435 mg/day (total flavonoids)	Finnish men without previous CHD	1950	Reduced risk of CVD death	(Mursu et al., 2008)
	Flavonol, flavan-3-ols				Reduced risk of ischemic stroke	
General diet	Flavonol	18 mg/day	Finnish male smoker aged 50-69	25,372	Reduced risk of non-fatal MI	(Hirvonen et al., 2001)
	Flavone					
General diet	Luteolin	0.2 mg/day	Finish men and women aged 65-99	755	Reduced risk of acute MI	(Marniemi et al., 2005)
	Kaempferol	1.3 mg/day				
General diet	Kaempferol	4.7 mg/day	American females aged 30-55	66,360	Reduced risk of CHD	(Lin et al., 2007)
General diet	Quercetin	28.6 mg/day (total	Elderly men from The	552	Reduced risk of stroke	(Keli et al.,

Polyphenol Source	Polyphenol	Dose	Study population	No. participants	Outcome at follow up	Ref.
		flavonoids)	Netherlands			1996)
General diet	Quercetin	3.3 mg/day	General population of Finland	10,054	Reduced mortality from cardiac ischemia	(Knekt et al., 2002)
	Kaempferol, naringenin, hesperetin	0.6, 5.1, 15.1 mg/day			Reduced stroke incidence	
General diet	Anthocyanidin, flavanones	0.2, 93.7 mg/day	Post-menopausal American women	34,489	Reduced risk of CHD, CVD or both	(Mink et al., 2007)
Tea, apples, chocolate	Catechin	72 mg/day	Elderly men from the Netherlands	806	Reduced ischemic heart disease mortality	(Arts et al., 2001b)
Olive oil	Hydroxytyrosol derivatives, flavonoids, lignans,	301 mg/day total polyphenols	American men and women over 18 years old with early atherosclerosis	82	Improved endothelial function	(Widmer et al., 2013)
Red grapefruit	Flavonoids, anthocyanins	One per day	Polish patients with coronary atherosclerosis	92	Reduced LDL, triglycerides, and total cholesterol	(Gorinstein et al., 2006)

CVD = cardiovascular disease; CHD = coronary heart disease; MI = myocardial infarction; LDL = low-density lipoproteins

Table 1.3: Table summarising the current literature on the anti-atherosclerotic effect of polyphenol rich products and extracts in the ApoE-/- mouse model

Diet type	Polyphenol containing product	Main polyphenol	Dose/delivery	Intervention duration	Location of plaque	Effect size (% inhibition)	Ref.
HF diet	Apple peel	Quercetin	20 % in diet	20 weeks	Thoracic aorta	Not quantified	(Gonzalez et al., 2015)
Western diet	Cocoa powder	Not stated	0.2 % in diet	12 weeks	Aortic sinus	47 %	(Guan et al., 2016)
			2 % in diet			32 %	
Normal diet	Pine bark extract	Proanthocyanidins	2 % in diet	9 weeks	Aortic sinus	~35 %	(Sato et al., 2009)
HF/HC diet	Dealcoholised red wine	Not stated	10 % in water	26 weeks	Thoracic aorta	~40 %	(Waddington et al., 2004)
Western diet	Apple polyphenols	Not stated	100 mg/kg/day	12 weeks	Aortic sinus	61 %	(Xu et al., 2015)
Normal diet	Pomegranate juice	Punicalagin/punicalin	200 µg GAE/day	3 months	Aortic sinus	~40 %	(Aviram et al., 2008)
HF/HC diet	Tea extract	Epigallocatechin gallate	0.8 g/L in sucrose	14 weeks	Aortic sinus	23 %	(Miura et al., 2001)

GAE = gallic acid equivalent; HF = high fat; HC = high cholesterol

Table 1.4: Table summarising the current literature on the anti-atherosclerotic effects of individual polyphenol compounds in the ApoE-/- mouse model

Diet type	Compound	PP dose/delivery	Intervention duration	Location of plaque	Effect size (% inhibition)	Ref.
HC diet	EGCG	10 mg/kg daily PP I.P. injections	6 weeks	Aortic sinus	Day 21: 55 % Day 42: 73 %	(Chyu et al., 2004)
HF diet	EGCG	0.8 g/L in water	7 weeks	Aortic sinus	Not quantified	(Yin et al., 2016)
Normal diet	Catechin	0.02 % in diet	6 weeks	Aortic sinus	32 %	(Auclair et al., 2009)
Normal diet	Quercetin	1.3 mg/day in diet	26 weeks	Aortic sinus	80 %	(Loke et al., 2010)
	Theaflavin				60 %	
Western diet	Catechin + caffeic acid + resveratrol	160 mg/kg body weight/day	8 weeks	Aortic sinus	40 %	(Norata et al., 2007)
				Ascending aorta	36 %	
HF diet	Quercetin	0.05 % in diet	14 weeks	Aortic sinus	20 %	(Shen et al., 2013)
				Thoracic aorta	54 %	
Normal diet	Protocatechuic acid	0.003 %	20 weeks	Aortic sinus	42 %	(Wang et al., 2010)
HF diet	Chlorogenic acid	400 mg/kg body weight/day	12 weeks	Aortic sinus	52 %	(Wu et al., 2014)
Normal diet	Caffeic acid phenethyl ester	30 mg/kg body weight/day	12 weeks	Thoracic aorta	~65 %	(Hishikawa et al., 2005)

PP = polyphenol; I.P = intraperitoneal; HF = high fat; HC = high cholesterol;

1.8 The VEGF protein

Vascular endothelial growth factor (VEGF) is the growth factor primarily responsible for angiogenic stimulation in the endothelial cells of humans and other animals (Ferrara, 2009). The VEGF protein functions as a dimer of around 45kDa size (Ferrara and Henzel, 1989), binding to a family of tyrosine kinase receptors known as the VEGF receptors, as well as the co-receptors neuropilin 1 and 2 (NRP1 and NRP2) and extracellular complexes such as heparin (Ferrara et al., 2003). There are multiple forms of VEGF with VEGFA being the most crucial as it is responsible for the majority of angiogenesis signalling and maintenance of vascular health, signalling via VEGFR-2 (Shalaby et al., 1995). Neither VEGFA heterozygous or homozygous negative mice survive past gestation due to defective formation of the vasculature demonstrating the importance of VEGFA in developmental blood vessel generation (Carmeliet et al., 1996, Ferrara et al., 1996). Due to its dominant role VEGFA is often referred to in the literature simply as VEGF, as will be done in this chapter, however a number of other VEGF ligands exist. VEGFB plays some role in regulating and maintaining the formed vasculature (Aase et al., 2001, Bellomo et al., 2000) and recently has been linked to neurogenesis in rodent models (Sun et al., 2006). VEGFC and VEGFD predominantly induce lymphangiogenesis through signalling via VEGFR-3. VEGFC and VEGFD have been shown to bind to VEGFR-2 following proteolysis albeit at a lower binding affinity than VEGFA (Joukov et al., 1997, McColl et al., 2007). Two further biological VEGF proteins have been documented, VEGFE and VEGFF, however they are produced by the parapox virus (Ogawa et al., 1998) and in eastern cottonmouth snake venom (Yamazaki et al., 2005) respectively. They are capable of inducing VEGF signalling via VEGFR-2 to varying levels of success.

1.9 The different VEGFA isoforms

The key VEGF protein, VEGFA, exists as five isoforms, produced from alternative splicing of the VEGF gene with one further isoform after proteolysis (Ferrara, 2010). These isoforms are $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{209}$, while $VEGF_{110}$ can be formed through proteolysis. The number refers to the number of amino acids in the protein. Each of these isoforms has a varying ability to induce signalling

via the VEGF receptors. The VEGF isoforms which are able to effectively induce the VEGF signalling pathway are those with only an intermediate binding affinity for the neuropilin co-receptors and the extracellular matrix component heparin. This affinity is dependent on inclusion of exon 6 and 7 in the splice variant (Krilleke et al., 2009). $VEGF_{121}$ and $VEGF_{110}$ lack both exon 6 and 7, are found freely in circulation and are poor inducers of VEGF receptor phosphorylation. $VEGF_{189}$ and $VEGF_{209}$, containing both exon 6 and 7, have a very strong affinity for heparin and are rapidly sequestered from circulation. Alternative splicing produces two VEGF isoforms with only one of these exons, $VEGF_{145}$ and $VEGF_{165,}$ including only exon 6 and 7 respectively (Dehghanian et al., 2014). $VEGF_{145}$ has specifically been shown to bind in a heparin in-dependent manner leading to a tissue specific response in corneal endothelial cells (Stalmans et al., 2002). $VEGF_{165}$ is able to recognise the majority of VEGF receptors and co-receptors and is the predominant angiogenic circulating VEGF isoform. A summary of the VEGF isoforms is shown in Table 1.5.

1.10 The VEGF receptors

The VEGF receptors are a family of tyrosine kinase receptors which have a distinct structure. The extracellular domain contains 7 immunoglobulin like domains which form the VEGF binding region (Shinkai et al., 1998, Fuh et al., 1998). The intracellular region of the receptor consists of multiple domains including, a juxtamembrane domain, two kinase domains separated by a kinase insert domain and a c-terminus. Until recently, it was thought that upon binding of the VEGF ligand a receptor dimer was formed leading to a series of phosphorylation events stimulating a variety of downstream signals. However, evidence is now emerging that the receptors may be able to form receptor dimers without the presence of the VEGF ligand with low levels of activity which only become fully active upon VEGF ligand binding and a conformational change in the transmembrane region (Sarabipour et al., 2016). Upregulation of ligand independent signalling can occur in diabetes mellitus patients due to an increase in reactive oxygen species (ROS) generation (Warren et al., 2014). There are 3 main VEGF receptors known as VEGFR-1, VEGFR-2 and VEGFR-3 and the receptor dimer pair can be both a homo-

or hetero-dimer and each combination produces an individual phosphorylation pattern (Nilsson et al., 2010, Neagoe et al., 2005, Cudmore et al., 2012).

1.10.1 VEGF receptor 1

VEGFR-1 receptor is stimulated by the VEGFA, VEGFB and platelet derived growth factor (PDGF) ligands. VEGFR-1 -/- mice die at embryonic day 8.5-9.0 due to an increase in endothelial progenitor cells causing a disorganised vascular network (Fong et al., 1995, Fong et al., 1999) suggesting that a lack of VEGFR-1 induces over commitment of progenitor cells to endothelial cell formation and not a lack of vessel formation. It is thought VEGFR-1 plays more of a regulatory role in angiogenesis by sequestering the circulating VEGF, controlling the amount available for signalling via VEGFR-2 (Fong et al., 1999, Hiratsuka et al., 1998). VEGFR-1 tyrosine kinase domain knockout mice, which have the VEGF binding region but do not generate the downstream signalling response, develop normal vasculature (Hiratsuka et al., 1998) but have impaired VEGF-induced macrophage migration, suggesting VEGFR-1 signalling plays a much large role in this function. A soluble form of VEGFR-1 has also been shown to behave as a sink for VEGF, reducing its mitogenic potential (Kendall and Thomas, 1993) and causing endothelial dysfunction in preeclampsia patients (Maynard et al., 2003). Interestingly, heterodimers of VEGFR-1 and VEGFR-2 can induce an individual vasodilatory response by releasing prostaglandin following activation (Neagoe et al., 2005).

1.10.2 VEGF receptor 2

VEGFR-2 is predominantly found as a homodimer and is the primary receptor responsible for VEGF stimulated angiogenesis and neoangigoenensis in the body, including pathological angiogenesis (Goodlad et al., 2006, Doi et al., 2012) as well as the response of endothelial cells to VEGF stimulation *in vitro* (Endo et al., 2003).The importance of VEGFR-2 is demonstrated in knockout mouse studies where VEGFR-2 -/- mice do not develop past embryonic day 8.5-9.5 due to lack of endothelial cell development and lack of blood vessel generation (Shalaby et al., 1995).

1.10.3 VEGF receptor 3

VEGFR-3 has demonstrated a key role in lymphangiogenesis (Dumont et al., 1998) and, in contrast to VEGFR-1 and VEGFR-2, VEGFR-3 cannot bind with VEGFA and instead binds with VEGFC and VEGFD. The exception to this is with a VEGFR-2/VEGFR-3 heterodimer which can bind VEGFA, VEGFC and VEGFD. The sites that become phosphorylated with VEGFR-2/VEGFR-3 heterodimer activation by a VEGF ligand seem to be predominantly associated with kinase regulation however evidence does suggest this heterodimer is found at a higher level in the tip cells of filopodia and may have a role in angiogenic sprouting (Deng et al., 2015, Nilsson et al., 2010).

Table 1.5: A summary of the different VEGF isoforms, the genetic differences between those isoforms and their physiological properties.

Table originally published by Edwards and Kroon (2013) in Recent Advances in Polyphenol Research Volume 4 with permission from John Wiley and Sons. .

VEGF Isoform	Exon 6	Exon 7	Extracellular matrix (ECM) binding	Receptor recognition	Functionality
121	X	X	No binding. Found in circulation	VEGFR-2	Poor functionality due to lack of heparin binding
145	✓	X	Intermediary binding to ECM	VEGFR-2	Able to activate VEGFR-2 but not any other receptors
165	X	✓	Intermediary binding to ECM	VEGFR-2, VEGFR1, Neuropilins	The predominant isoform, with intermediary heparin binding
189	✓	✓	Strong binding to ECM	N/A	Rapidly sequestered due to high affinity for heparin. Not seen in circulation
209	✓	✓	Strong binding to ECM	N/A	Rapidly sequestered due to high affinity for heparin. Not seen in circulation
110	X	X	No binding. Found in circulation	VEGFR-2	Acts as VEGF121 with poor functionality due to lack of heparin binding

1.11 The stimulated VEGFR-2 signalling pathway

Each phosphorylation site identified on VEGFR-2 has a distinct signalling role leading to the range of physiological outcomes that allow for vessel growth, permeability, cell survival and migration. Tyrosine 1054 and 1059 residues are highly conserved across all 3 VEGF receptors due to their integral role in the kinase activation loop. Without activation of these phosphorylation sites only 10% of the receptor activation is able to occur, significantly impacting on VEGFR-2 signalling capability (Dougher and Terman, 1999). These phosphorylation sites are further controlled by the intracellular juxtamembrane region where, if this region in VEGFR-1 and VEGFR-2 are swapped, VEGFR-2 has reduced receptor kinase activation while receptor activity is significantly increased in VEGFR-1, suggesting the juxtamembrane region plays a role in the kinase activation (Gille et al., 2000).

1.11.1 Tyrosine 951, 1054 and 1059 signalling

The kinase insert domain is also important for VEGF mediated angiogenesis as replacing the Y951 in this region with a phenylalanine residue stops Y1175 phosphorylation associated downstream signalling (Manni et al., 2014) .The tyrosine 951 site activates the signalling molecule T-cell specific adapter protein (TSAd), leading to membrane bound Src signalling and induced vascular permeability (Sun et al., 2012). TSAd stimulation of Src has been shown to lead to phosphorylation of another receptor tyrosine kinase, Axl, which stimulates phosphorylation of AKT signalling independent of Y1175 phosphorylation (Ruan and Kazlauskas, 2012). Src has also been shown to bind to phosphorylated Y1057 (Y1059 in humans) and leads to phosphorylation of the key angiogenesis Y1173 (Y1175 in humans) and facilitates the phosphorylation of IQGAP1 which, in turn leads to b-Raf stimulated proliferation in endothelial cells (Meyer et al., 2008). Src also has an interesting role in positive feedback following VEGF stimulation where VEGFR-2^{+}/Src^{+} intracellular vesicles are mobilised to the cell membrane providing a new supply of VEGFR-2 for stimulation with close proximity to the Src binding partner (Gampel et al., 2006). A summary of the signalling pathways stimulated by VEGF stimulated phosphorylation of the VEGFR-2 tyrosine 951, 1054, and 1059 residues can be seen in Figure 1.7

1.11.2 Tyrosine 1175 signalling

Tyrosine 1175 is known to be the key phosphorylation site for the majority of angiogenesis signalling by the VEGFR-2 receptor as transgenic mice for mutated Y1175 die at embryonic day 8.5-9, in almost an identical manner to mice completely deficient in the VEGFR-2 receptor (Sakurai et al., 2005a). Perhaps the dependency of functional angiogenesis on stimulation of the VEGFR-2 Y1175 site provides an explanation for the large number of regulatory pathways associated with this phosphorylation event. The molecule Shb is phosphorylated in a Src dependent manner after binding to the phosphorylation Y1175 site and stimulates focal adhesion kinase (FAK) phosphorylation (Holmqvist et al., 2004) which mediates vascular permeability (Chen et al., 2012). Furthermore, phosphoinositol-3-kinase (PI3K) activation is dependent on the presence of FAK (Qi and Claesson-Welsh, 2001). Considering the presence of Shb has been demonstrated to be important for the VEGF stimulated activation of PI3K (Holmqvist et al., 2004) it could be suggested that Shb mediates PI3K activity through FAK. PI3K facilitates phosphorylation of Akt (Cantley, 2002) which, in turn reduces the activity of apoptotic signalling molecules (Cardone et al., 1998, Datta et al., 1997, Trinh et al., 2009). Akt can also phosphorylate endothelial nitric oxide synthase (eNOS) (Fulton et al., 1999) to stimulate vascular permeability (Duran et al., 2010). The phosphorylated Y1175 site also provides the binding site for phospholipase C gamma (Plc-γ) (Sawano et al., 1997, Takahashi and Shibuya, 1997, Takahashi et al., 2001) and stimulated an elevation in intracellular calcium which correlates to the stimulation of mitogen activated protein kinase (MAPK) signalling and cell proliferation (McLaughlin and De Vries, 2001) and eNOS production of nitric oxide (NO) (Gélinas et al., 2002). Furthermore, Plc-γ stimulates the production of inositol-3-phosphate (IP_3) and diacylglycerol (DAG) which stimulate protein kinase C (PKC) leading to MAPK induction of platelet activating factor induced vascular permeability (Sirois and Edelman, 1997, Bernatchez et al., 1999) and to increased gene expression and cell proliferation (Xia et al., 1996). Sck has also been shown to bind at Y1175 and caused a reduction in MAPK phosphorylation although the physiological outcome of this has not been further explored (Ratcliffe et al., 2002). A summary of the signalling

pathways stimulated by VEGF stimulated phosphorylation of VEGFR-2 tyrosine 1175 residue can be seen in Figure 1.8.

1.11.3 Tyrosine 1214 signalling

Y1214, another C-terminal region tyrosine, has been highlighted as an important phosphorylation site for VEGFR-2 signalling. VEGFR-2 stimulation with VEGF and β1 integrin results in a prolonged phosphorylation at this site. Phosphorylation at Y1214 causes recruitment of the protein Fyn while recruitment of Nck occurs in a Fyn dependent manner (Lamalice et al., 2006). p21-activated kinase 2 (PAK-2) is also phosphorylated in a Fyn and p-Y1214 dependent manner (Lamalice et al., 2006) to stimulate SAPK2/p38 actin remodelling and endothelial migration (Lamalice et al., 2004). Furthermore, Y1214 appears to be important in differentiating the signal transduction between matrix bound VEGF and free VEGF with matrix bound VEGF associated with a prolonged stimulation of p38 MAPK (Chen et al., 2010, Wang et al., 2013). Heat shock protein 90 (HSP90) has also been shown to interact with the 130 amino acid residues of the c-terminal end of VEGFR-2. HSP90 also associates with αvβ3 integrin to stimulate a VEGFR-2-αvβ3 integrin dependent activation of FAK through the signalling molecule RhoA and RhoA kinase (ROCK) (Le Boeuf et al., 2004) leading to angiogenesis associated cell migration (Bryan et al., 2010, Haskell et al., 2003, Avraham et al., 2003). A summary of the signalling pathways stimulated by VEGF stimulated phosphorylation of VEGFR-2 tyrosine 1214 residue can be seen in Figure 1.9.

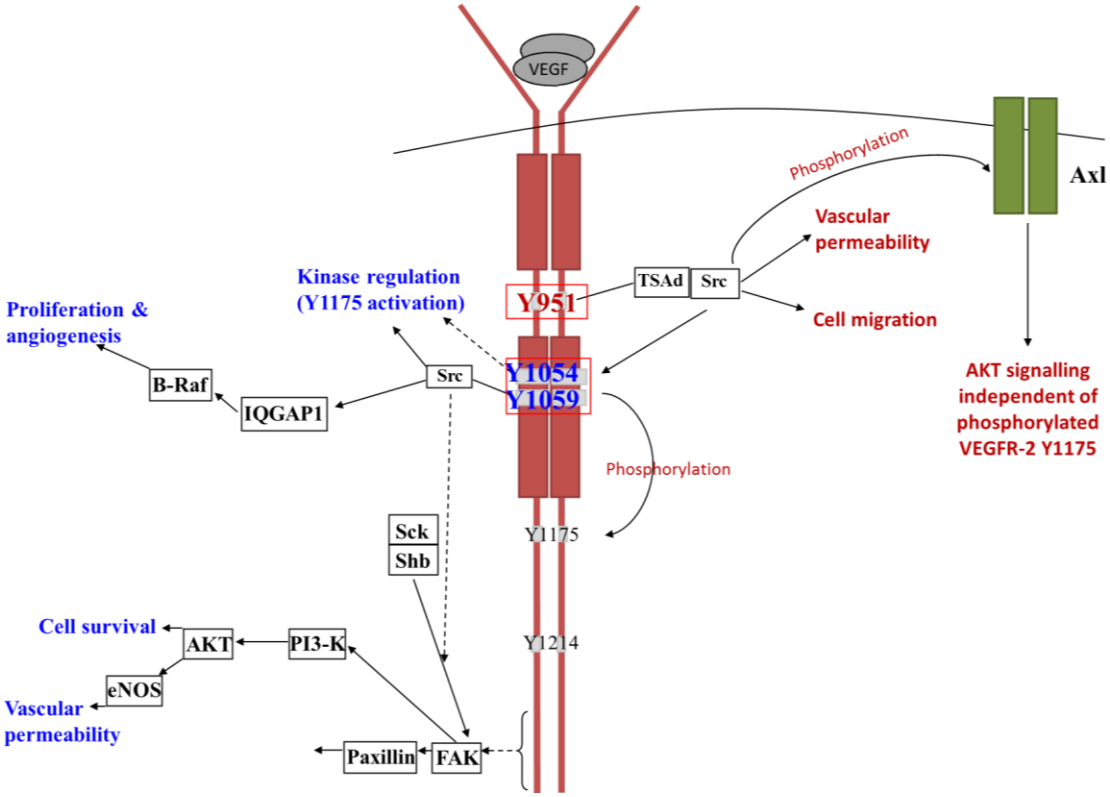


Figure 1.7: VEGF stimulated VEGFR-2 signalling from the phosphorylated tyrosine 951, 1054 and 1059 residues

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Abbreviations: AKT = protein kinase B; Axl = Tyrosine-protein kinase receptor UFO; B-Raf = B-Raf proto-oncogene; eNOS = endothelial nitric oxide synthase; FAK = Focal adhesion kinase; IQGAP1 = Ras GTPase-activating-like protein IQGAP1; PI3-K = Phosphoinositide 3-kinase; Sck = Shc-related adaptor protein; Shb = SH2 domain-containing adapter protein B; Src = Proto-oncogene tyrosine-protein kinase Src; TSAd = T cell-specific adapter; Y = tyrosine.

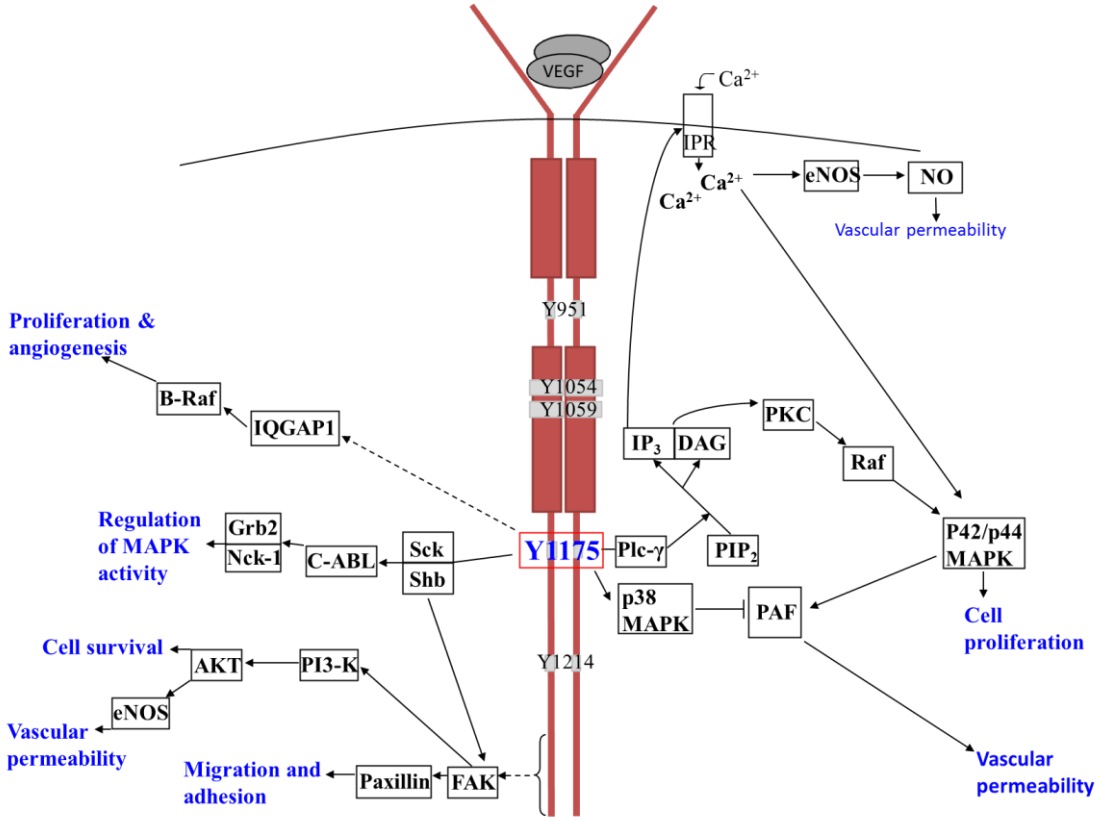

Figure 1.8: VEGF stimulated VEGFR-2 signalling from the phosphorylated tyrosine 1175 residue

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Abbreviations: AKT = protein kinase B; B-Raf = B-Raf proto-oncogene; C-ABL =Abelson murine leukemia viral oncogene homolog 1; Ca2+ = calcium; DAG = diacylglycerol; eNOS = endothelial nitric oxide synthase; FAK = Focal adhesion kinase; Grb2 = Growth factor receptor-bound protein 2; IP3 = inositol triphosphate; IPR = IP3 receptor; IQGAP1 = Ras GTPase-activating-like protein IQGAP1; MAPK = mitogen activated protein kinase; Nck-1 =NCK adaptor protein 1; NO = nitric oxide; PAF = Platelet-activating factor; PIP2 = Phosphatidylinositol 4,5-bisphosphate; PI3-K = Phosphoinositide 3-kinase; PKC = protein kinase C; Plc-γ = Phospholipase C gamma; Raf = rapidly accelerated fibrosarcoma; Sck = Shc-related adaptor protein; Shb = SH2 domain-containing adapter protein B; Src = Proto-oncogene tyrosine-protein kinase Src; TSAd = T cell-specific adapter; Y = tyrosine

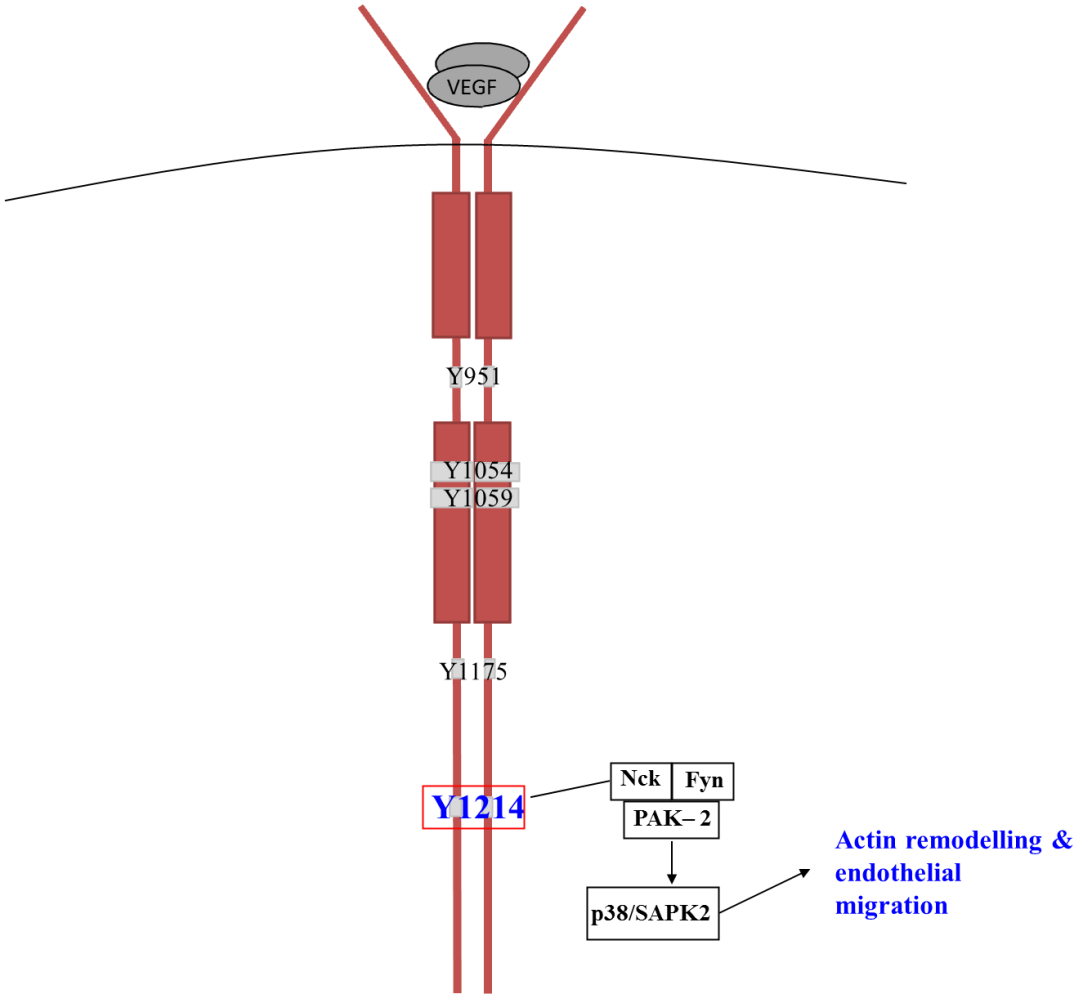


Figure 1.9: VEGF stimulated VEGFR-2 signalling from the phosphorylated tyrosine 1214 residue

Edited from Chapter 9 Recent Advances in Polyphenol Research Volume 4; (Edwards and Kroon, 2014) with permission from John Wiley and Sons.

Abbreviations: Nck-1 =NCK adaptor protein 1; Fyn = Proto-oncogene tyrosine-protein kinase Fyn; PAK-2 = Serine/threonine-protein kinase PAK 2; SAPK2 = Serine/threonine-protein kinase SAPK2; Y = tyrosine.

1.12 Disease relevance of VEGF signalling

VEGF signalling and the associated angiogenesis response is necessary in both development and regulation of the vascular system (Shalaby et al., 1995, Carmeliet et al., 1996, Ferrara et al., 1996). The VEGF signalling pathway is highly controlled (Simons et al., 2016) however abnormal regulation of VEGF signalling can lead to pathological angiogenesis (Goodlad et al., 2006, Doi et al., 2012, Carmeliet, 2005) or vascular dysfunction (Maynard et al., 2003, Robinson et al., 2010).

1.12.1 Diseases currently treated by anti-VEGF medication

Upregulation of the VEGF signalling pathway has been identified as a crucial step in the development (Mattern et al., 1996, Kopparapu et al., 2013) and metastasis (Takahashi et al., 1995, Weis et al., 2004) of numerous cancers. Circulating VEGF levels are increased in certain metastatic cancers (Duque et al., 1999) and have been suggested as a prognostic factor for certain cancers (Kim et al., 2003). Hypoxia caused through rapid tissue expansion is also known to stimulate tumour angiogenesis as well as VEGF expression (Liao and Johnson, 2007, De Francesco et al., 2013). Upregulation of VEGF signalling (Jensen et al., 2006) in the tumour and surrounding stromal cells (Fukumura et al., 1998) also increases tumour angiogenesis (Rak et al., 1995). Bevacizumab and ranibizumab (Yang et al., 2003, Saltz et al., 2008, Giantonio et al., 2007), VEGF targeting monoclonal antibody treatments, and sorafenib, sunitinib and pazopanib (Bergh et al., 2012, Escudier et al., 2009, Cheng et al., 2013, Sternberg et al., 2010), multikinase inhibitors known to affect the VEGF receptors, have been found to be highly effective alone and in combination therapies at reducing a range of metastatic cancers. VEGF is also associated with wet age-related macular degeneration. Unnecessary blood vessel generation in the tissues of the eye stimulated by increased VEGF expression (Kvanta et al., 1996, Kliffen et al., 1997) results in fibrosis and a loss of vision clarity (Friedlander, 2007). The anti-VEGF therapy ranibizumab has been approved for use in the NHS for treatment of this VEGF associated macular degeneration (Rosenfeld et al., 2006, Brown et al., 2009).

1.12.2 Side effects of anti-VEGF treatments

In recent years there have been reports of side-effects of anti-VEGF therapies. However, a recent meta-analysis of intravitreal monoclonal anti-VEGF treatments did not associate the treatment with increased CVD events or mortality risk, although there was a link with venous thromboembolic events and non-ocular haemorrhage (Thulliez et al., 2014). Bevacizumab treatment of cancer has also been associated with an increased risk of cardiac ischemia and thromboembolic events (Ranpura et al., 2010) while sorafenib is associated with an increased risk of hypertension (Wu et al., 2008) although the reason is not understood. This is perhaps not overly unexpected as VEGF plays an important role in inducing vasodilation and hypotension via NO release (Yang et al., 1996) however it raises extra considerations when prescribing the treatment. Anti-VEGF monoclonal antibody bevacizumab has been predicted by pharmacokinetic modelling to induce higher circulating VEGF levels after intravenous (I.V.) injection, hypothesised to be due to reduced VEGF clearance after binding within the monoclonal antibody which may explain the thrombotic risk associated with such treatment, although this is only identified with Bevacizumab in particular (Stefanini et al., 2010). Furthermore the pan-VEGFR tyrosine kinase inhibiting treatments (Ebos et al., 2009) as well as VEGFR-2 specific inhibitors (Pàez-Ribes et al., 2009) have been found to accelerate tumour metastasis in animal models. There has not been reported increases in metastasis in the clinical trials of anti-VEGF treatments although anti-VEGF treatment of mice transplanted with human glioblastoma cells had reduced tumour blood vessel density but increased hypoxia induced invasion into healthy tissue (Keunen et al., 2011) suggesting that further understanding of the effects of VEGF inhibition is needed.

1.12.3 Association of VEGF with cardiovascular disease

Increasing evidence suggests that growth factors and particularly VEGF or its associated signalling pathways contribute to atherosclerosis (Blann et al., 2002, Fleisch et al., 1999). Angiogenesis and neovascularisation have been known to play a role in cardiovascular disease for some time (Khurana et al., 2005). Under normal conditions the inner lining of the artery wall, the intima, is supplied sufficient

nourishment without its own specific network of vessels. However, this capacity is limited and during atherosclerotic development as the intima expands beyond this diffusible distance resulting in a loss of the supply of the necessary compounds for growth, including oxygen, to the core of these tissues. This stimulates new blood vessel generation from the vasa vasorum, the network of vessels supplying bigger vessels, to support the growing plaque (Bjornheden et al., 1999) The generation of these vessels is linked with an upregulation of hypoxia inducible factor 1α (HIF1α) (Kuwahara et al., 2002), a known activator of VEGF transcription (Hirota and Semenza, 2006), and allows for the continual supply of the building blocks of the atherosclerotic plaque. In both humans and mice the link between increased atherosclerotic disease and vessel wall neovascularisation is demonstrated by an increase in blood supply to the aortic tissue correlating to characteristics of advancing atherosclerosis, such as necrotic core formation (Hiyama et al., 2010, Juan-Babot et al., 2003, Fleiner et al., 2004) and increased risk of plaque vulnerability and rupture (Virmani et al., 2005). In the ApoE-/- model known angiogenesis inhibitors significantly reduced the atherosclerotic lesion size by up to 85 % (Moulton et al., 1999). Following a review of plasma VEGF levels in the Framingham study participants (the largest continuous CVD epidemiological study to date (Dawber et al., 1951)) demonstrated that those in the second and third quartiles of plasma VEGF levels had an increased risk of CVD of 34 % and 59 % respectively compared to the first quartile (Kaess et al., 2016) although, interestingly, in the fourth quartile with the highest VEGF levels this association was not observed. Another study concluded VEGF serum concentration could become a useful prognostic factor for atherosclerosis due to correlation with CVD risk factors, particularly in men (Kimura et al., 2007). Although it is important to note that not all studies have observed this strong link (Sandhofer et al., 2009, Alber et al., 2005) an increase in intra-plaque VEGF and VEGFR-2 levels have been associated with neovascularisation and greater macrophage density of the atherosclerotic plaque (Ma et al., 2016, Inoue et al., 1998, Chen et al., 1999). Plasma VEGF levels have also been compared with patient angiograms, showing VEGF levels were significantly higher in the critical lesion group, but not the non-critical lesion group, when compared to the control (Kucukardali et al., 2008). This supports the notion that

angiogenesis is important during advancing and dangerous atherosclerosis and may offer an explanation why contradictory reports on the relevance of circulating VEGF levels are found in the literature when all levels of disease are taken into account. Diseases such as type-I diabetes (Iacobellis et al., 2004) and systemic lupus erythematosus (SLE) (Colombo et al., 2009) are associated with increased risk of CVD and have increased VEGF levels which, in the case of SLE, was associated with increased intima media thickness (IMT) of the carotid artery.

1.12.4 The role of VEGF in atherosclerosis

Direct evidence of the stimulatory action of VEGF on atherosclerotic plaque growth has been observed in ApoE-/- $ApoB^{100}$-/- mice and New Zealand white rabbits. The plaque area in the aortic arch was significantly increased following a single intraperitoneal injection of VEGF versus a serum albumin control. The VEGF injection also induced greater macrophage infiltration and vascularisation of the plaque (Celletti et al., 2001a, Celletti et al., 2001b). Similarly in the ApoE-/- model, but not other cholesterol driven atherosclerosis models, adenoviral gene transfer caused high systemic VEGF expression levels and induced the progression of atherosclerosis (Heinonen et al., 2013). Using the VEGF injected rabbit model, it was also demonstrated that the VEGF induced progression of atherosclerotic disease could be significantly reduced by paclitaxel and angiostatin treatment (Celletti et al., 2002). Together, these reports demonstrate that VEGF, and specifically the $VEGF_{165}$ isoform, is capable of driving atherosclerosis progression by increasing the angiogenic potency of vascular tissues, and that anti-angiogenic therapies can block the pro-atherosclerotic action of VEGF. Vaccination against VEGFR-2 resulted in a 77% reduction in the formation of new lesions and limited progression of atherosclerosis by 66% in hypercholesterolaemic models (Hauer et al., 2007, Petrovan et al., 2007) suggesting the angiogenic actions of VEGF through VEGFR-2 are associated with its atherogenic potential. However, in the ApoE-/- mouse model a pan-VEGF receptor inhibitor which blocks not only the actions of VEGFR-2 but of VEGFR-1 and VEGFR-3 as well (Winnik et al., 2013) significantly increased atherosclerotic plaque area in comparison to controls. Perhaps this is due to the key role of VEGF in endothelial maintenance (Horowitz et al., 1997) and

blood vessel generation following injury (Couffinhal et al., 1999) as there is a risk that complete inhibition of VEGF activity could negatively impact cardiovascular health, as has been suggested is the case for anti-VEGF monoclonal treatments for other diseases. Nevertheless, these studies demonstrate the need for further investigation into the role of VEGF in atherosclerosis but suggest that regulation of raised VEGF levels may still be an important avenue of investigation as treatment for atherosclerotic disease.

1.13 VEGF signalling within the atherosclerotic plaque

VEGF signalling is a necessity for normal vascular health and critical in new vascular development (Carmeliet et al., 1996). Given this critical role it is regulated in a complex manner with many different cell types being able to secrete VEGF (Pertovaara et al., 1994, Taichman et al., 1997, Namiki et al., 1995), including macrophages (McLaren et al., 1996). In pathological angiogenesis VEGF signalling can be initiated in an autocrine (Soker et al., 2001), paracrine (Villegas et al., 2005) or intracrine (Bhattacharya et al., 2016) manner. Circulating VEGF levels are higher in patients with severe atherosclerotic lesions (Kucukardali et al., 2008) and VEGF levels in tissue are found to be higher in atherosclerotic plaque sites (Ho-Tin-Noe et al., 2011) demonstrating that VEGF is not only raised in systemic circulation (Kimura et al., 2007, Blann et al., 2002, Fleisch et al., 1999) but also localised within the plaque itself. The morphology of atherosclerosis may provide insight into why raised VEGF levels are associated with atherosclerotic plaque. One of the earliest stages of atherosclerosis development is the build-up of oxidised low density lipoprotein (OxLDL) laden macrophages (foam cells) in the artery wall. The uptake of OxLDL has been shown to stimulate macrophages to release VEGF protein (Riazy et al., 2009) and to cause macrophage associated accumulation of VEGF in the atherosclerotic plaque (Ramos et al., 1998, Inoue et al., 2001) even at the early stages of plaque formation. Macrophages have also demonstrated a novel role in vascular tip cell fusion and therefore creating new vascular networks (Fantin et al., 2010). A high cholesterol diet is associated with VEGF expressing macrophage induction of angiogenesis in atherosclerosis (Hutter et al., 2013). Further recruitment of inflammatory cells occurs in atherosclerosis through secretion of

chemokines by plaque associated tissues (Wezel et al., 2015). The immune system is also plays a role in regulating pathological VEGF-induced angiogenesis in retinal vascular related disease with some cases requiring immune suppression and anti-VEGF treatment to limit angiogenic progression (Sene et al., 2015).

Hypoxia is also able to induce recruitment of neutrophils to the atherosclerotic plaque resulting in pro-angiogenic expression of MMP9 which encourages release of VEGF from the extracellular matrix within the plaque (Christoffersson et al., 2012). Equally hypoxia is known be present within the atherosclerotic plaque as HIF1α expression was seen within endothelial cells of lesions in the ApoE-/- mouse model (Akhtar et al., 2015). VEGF expression is directly induced by HIF1α as it binds to the promoter region of the VEGF gene and directly increases VEGF protein expression (Liu et al., 1995).

Extracellular matrix components form a critical part of developing atherosclerosis, for example fibronectin is associated with wound repair in the developing plaque and is found in up 80 % of early plaques in humans (Kakolyris et al., 1995). It is also capable of acting as a VEGF store and being associated with promotion of new vascular networks though orchestrating VEGF gradients (Ruhrberg et al., 2002, Stenzel et al., 2011), with critical VEGF-induced branching not occurring when the interaction of VEGF and fibronectin is interrupted (Wijelath et al., 2002). Furthermore, the circulatory $VEGF_{121}$ is able to bind to the extracellular matrix in acidic conditions as induced in hypoxic tissues (Goerges and Nugent, 2004), and could therefore have the potential to upregulate VEGF signalling in fibronectin rich, hypoxic conditions (Reynolds et al., 2009). It could be suggested that VEGF activity would also be upregulated by the increase in fibronectin, collagen and other extracellular components of a growing atherosclerotic plaque, even from the early stages of plaque development.

Platelet aggregation and activation is associated with progressive and unstable atherosclerosis (Huo et al., 2003) as well as the release platelet derived VEGF (Wartiovaara et al., 1998). Platelet VEGF itself has been identified in the fibrin networks of atherosclerotic thrombotic tissues (Arisato et al., 2003) with platelet aggregation also resulting in the release of pro-angiogenic compounds such as

MMP's (Sawicki et al., 1997) which have been shown to induce the release of VEGF from the fibrinogen network in pathological angiogenesis (Belotti et al., 2003).

Furthermore, LDL can bind to VEGFR-1 and stimulate autophosphorylation, internalisation and degradation (Usui et al., 2007) while Apolipoprotein B has been shown to downregulate VEGFR-1 expression in hyperlipidaemic mice (Avraham-Davidi et al., 2012). This reduces the VEGFR-1 able to act as a sink for the VEGF ligand in epithelium rich in these compounds, as would be found in atherosclerotic plaque sites, which may result in more free VEGF available to stimulate VEGFR-2 associated angiogenic signalling.

1.14 Effect of polyphenols on VEGF signalling

1.14.1 Polyphenol effects on VEGF gene and protein expression

Determining VEGF and VEGF receptor gene and protein expression is one method by which the effects of polyphenols on VEGF signalling have been investigated. While there are a few reports of an increase in VEGF gene (Lin et al., 2010, Nicholson et al., 2010) and protein expression (Oak et al., 2006, Lin et al., 2010) a wide variety of polyphenols have demonstrated the opposite effect, eliciting strong inhibition of VEGF gene and protein expression, including rutin (Guruvayoorappan and Kuttan, 2007), quercetin (Oh et al., 2010, Pratheeshkumar et al., 2012), kaempferol (Luo et al., 2012, Luo et al., 2009), delphinidin, resveratrol (Dann et al., 2009), cyanidin (Oak et al., 2006), and EGCG (Lee et al., 2004, Dann et al., 2009) treatment. These studies focus on longer incubation times of between 3 and 24 hours predominantly in unstimulated cells however stimulation with hypoxic conditions, hydrogen peroxide (H_2O_2), or platelet derived growth factor have also been undertaken. The concentrations that have previously demonstrated an effect range from 1 - 100 µM. For example, at 30 µM a range of polyphenols all demonstrated inhibition of VEGF gene expression in unstimulated MCF-7 breast cancer cells following 18 hour treatment, but with a range of efficacies. Quercetin was the most effective (74 % inhibition) followed by silibinin (65 %), apigenin (47 %), morin (43 %), and naringenin (28 %). Conversely, 1 µM quercetin treatment of unstimulated HUVECs for 24 hours demonstrated an upregulation of VEGF gene

expression although treatment of HUVECs with the same dose of quercetin in combination with H_2O_2 for 24 hours caused a reduction in VEGF gene expression. Perhaps this suggests that quercetin inhibition of VEGF gene expression is only occurring in conditions where VEGF is upregulated such as cancer cells or after H_2O_2 treatment, a known VEGF stimulant (Cisowski et al., 2005). VEGFR-2 protein expression has also been shown to be downregulated in unstimulated SW837 adenocarcinoma cells following 24 hour incubation with 50 µM EGCG (Shimizu et al., 2010).

1.14.2 Polyphenol effects on VEGFR-2 phosphorylation

A variety of dietary polyphenols can affect VEGFR-2 phosphorylation. Delphinidin, cyanidin, the green tea catechins, scopoletin, ellagic acid, and wogonin among others have demonstrated inhibition of this key angiogenesis stimulating receptor (Huang et al., 2012, Lamy et al., 2006, Wen et al., 2008, Lamy et al., 2002, Lee et al., 2004, Liu et al., 2008, Neuhaus et al., 2004, Rodriguez et al., 2006, Lu et al., 2008, Shimizu et al., 2010, Pan et al., 2011, Cerezo et al., 2015, Moyle et al., 2015). These studies all differ in both the polyphenol incubation time which ranged from 5 minutes – 24 hours and the VEGF stimulation time which ranged from 0 – 10 minutes. The majority of these studies undertook long duration pre-treatment with the polyphenol prior to a short VEGF stimulation. Of the polyphenols tested in this manner some effects were observed at comparatively low concentrations, for example, delphinidin at 2 µM inhibited around 50 % VEGF stimulated VEGFR-2 phosphorylation in HUVECs following an 18 hour pre-treatment (Lamy et al., 2006). Ellagic acid inhibited 48 % VEGFR-2 phosphorylation in bovine aortic endothelial cells (BAECs) at 5 µM for the same pre-treatment duration of 18 hours (Labrecque et al., 2005). 60 minute pre-treatment of HUVECs with up to 100 µM EGCG significantly reduced the binding of labelled VEGF suggesting this pre-treatment blocked this interaction (Kondo et al., 2002) while *silico* modelling suggested that ellagic acid was predicted to bind the VEGFR-2 at the receptor binding region (Wang et al., 2012) suggesting a possible mechanism for the polyphenol inhibition of VEGF-induced VEGFR-2 phosphorylation. More recently however polyphenols have been shown to exert potent inhibition of VEGF-induced VEGFR-2 phosphorylation. In

these studies the polyphenol was mixed with VEGF for 5 minutes prior to being used to treat HUVECs for 5 minutes. The concentration required to elicit 50 % inhibition was used as a comparator. Many polyphenols demonstrated inhibition in this model at nanomolar or micromolar concentrations. The most potent of these were EGCG (88 nM), catechin gallate (94 nM), quercetagetin (96 nM), myrecetin (121 nM), epicatechin gallate (160nM), ellagic acid (230 nM), and procyanidin d.p 4 (280 nM). Furthermore, it was reported that while quercetin was able to inhibit 50 % VEGF-induced VEGFR-2 phosphorylation at 754 nM all quercetin glycosides tested were unable in inhibit at any concentration tested (Cerezo et al., 2015). It was also demonstrated that following pre-mixing of VEGF with EGCG, VEGF could not recover VEGFR-2 phosphorylation activity even after dialysis suggesting that the polyphenol was able to bind to the VEGF ligand to cause the observed inhibition (Moyle et al., 2015). This hypothesis was supported through *in silico* modelling which demonstrated a correlation between the predicted strength of binding to VEGF and the potency of inhibition of VEGFR-2 phosphorylation (Cerezo et al., 2015).

1.14.3 Polyphenol effects on downstream VEGF signalling partners

The effect of polyphenols on downstream VEGF signalling has also been investigated. ERK1/2 and MAPK inhibition is seen after treatment with a variety of diet derived polyphenols *in vitro* either by inhibiting phosphorylation or expression (Neuhaus et al., 2004, Luo et al., 2012, Pan et al., 2011, Kim et al., 2007b, Jung et al., 2009) although only resveratrol (Lin et al., 2003), ellagic acid (Labrecque et al., 2005) and EGCG (Neuhaus et al., 2004) have specifically demonstrated inhibition of VEGF-induced ERK phosphorylation. Interestingly 20 µM Kaempferol, apigenin or luteolin stimulation for differing lengths of time induced phosphorylation of ERK although none of these treatments used VEGF to induce phosphorylation (Luo et al., 2009, Lamy et al., 2012). NFκB protein levels and activation were observed to be lower in cells treated with kaempferol and EGCG respectively (Luo et al., 2012, Kim et al., 2007b). Furthermore, 16 weeks dietary intervention with Naringenin in rats reduced hepatic expression of NFκB (Subramanian and Arul, 2012). VEGF-stimulated phosphorylation of AKT and/or eNOS was also shown to be reduced in a

concentration dependent manner by EGCG (Rodriguez et al., 2006, Tang et al., 2003) and scopoletin (Pan et al., 2011) while general inhibition of AKT was demonstrated with acacetin, quercetin and kaempferol (Luo et al., 2009, Liu et al., 2011, Oh et al., 2010).

1.15 Effect of polyphenols on VEGF associated physiological responses

1.15.1 Polyphenol effects on VEGF associated cell signalling *in vitro*

The effect of polyphenols on the key physiological outcomes following VEGF stimulation has also been investigated. Cell proliferation can be inhibited by food extracts containing polyphenols such as grape seed extract or green tea extract (Kojima-Yuasa et al., 2003, Wen et al., 2008, Chen et al., 2011a) although these results must be interpreted with caution as there is no way to determine which component of the complex extract will be causing the effect. The effect if individual polyphenol treatment has also been investigated using for example the tea catechins EC, EGC, ECG, and EGCG, among others (Luo et al., 2012, Jung et al., 2009, Guruvayoorappan and Kuttan, 2007, Kondo et al., 2002). Naringenin at 1 µM was able to significantly reduce mouse aortic smooth muscle cell proliferation following 3 days of PP treatment (Chanet et al., 2012) while delphinidin and gallic acid have both increased the number of cells in cell cycle arrest after VEGF stimulation (Hseu et al., 2011, Favot et al., 2003). Cell migration has been inhibited by a wide variety of polyphenols e.g. resveratrol, quercetin, and delphinidin (Scoditti et al., 2012, Lamy et al., 2006) and decursin and wogonin extracted from plants (Lu et al., 2008, Jung et al., 2009). The green tea catechins each demonstrated different potential to inhibit VEGF-induced HUVEC migration, mirroring the results from the studies into inhibition of VEGFR-2 phosphorylation with the galloylated catechins proving more effective (Kondo et al., 2002).

1.15.2 Polyphenol effects on angiogenesis *in vitro*

The tube formation of HUVECs is a popular angiogenesis assay in the literature however comes with the limitation that it models vasculogenesis rather than angiogenesis. This model seeds vascular cells between two layers of an extracellular

matrix allowing them to grow over a set period of time after which the number of tubes or branch points is quantified. Scopoletin, delphinidin, resveratrol, and the green tea catechins have all been reported to inhibit VEGF-induced tube formation in this model (Pan et al., 2011, Lamy et al., 2006, Lin et al., 2003, Liu et al., 2008, Tang et al., 2003, Pratheeshkumar et al., 2012) while galloylation of the catechins proved an important structural aspect to increase potency (Kondo et al., 2002). Resveratrol was a particularly potent inhibitor in this model being able to inhibit over 50 % of VEGF induced tube formation at the low concentration of 1 µM (Lin et al., 2003). Other polyphenols have also demonstrated an inhibition of unstimulated tube formation including delphinidin and the green tea catechins as mentioned above (Labrecque et al., 2005, Lamy et al., 2006, Guruvayoorappan and Kuttan, 2007, Lamy et al., 2002, Kondo et al., 2002, Liu et al., 2008) although it should be noted that one study identified ferulic acid as a stimulant of tube formation (Lin et al., 2010). Ellagic acid was one of the polyphenols which demonstrated an inhibitory effect on endogenous tube formation at a supra-physiological concentration of 5 µM (Labrecque et al., 2005).

1.15.3 Polyphenol effect on angiogenesis *ex vivo*

The aortic ring assay involves embedding aortic tissue into an extracellular matrix and reporting the number of new vessel which grow, typically stimulated by VEGF. Quercetin, rutin, resveratrol, and ellagic acid among others (Chen and S. Easton, 2011, Wang et al., 2012, Pratheeshkumar et al., 2012, Guruvayoorappan and Kuttan, 2007, Matsubara et al., 2005) as well as a number of polyphenol rich extracts (Lu et al., 2010, Wen et al., 2008, Kang et al., 2013, Daleprane et al., 2012) have been able to inhibit new vessel generation from *ex vivo a*ortic tissue. Quercetin demonstrated anti-angiogenic at 20 µM concentrations and above (Pratheeshkumar et al., 2012) while rutin significantly inhibited angiogenesis at a 25 µg/ml concentration (Guruvayoorappan and Kuttan, 2007). Catechin however was not able to inhibit new vessel generation in the aortic ring assay (Negrão et al., 2013).

1.15.4 Polyphenol effects on angiogenesis *in vivo*

The chick chorioallantoic membrane (CAM) assay involves placing the polyphenol treatment onto the membrane just beneath a removed portion of the egg shell and the numbers of blood vessels or vessel branches are quantified as a measure of angiogenesis. Once again polyphenol rich extracts (Maiti et al., 2003, Toi et al., 2003) and a range of polyphenols were able to inhibit this angiogenesis (Pan et al., 2011, Pratheeshkumar et al., 2012, Oh et al., 2010, Hseu et al., 2011, Favot et al., 2003) with quercetin (Pratheeshkumar et al., 2012) and resveratrol (Brakenhielm et al., 2001) demonstrating inhibition of blood vessel formation albeit at high concentrations while ellagic acid was able to inhibit 90 % of VEGF stimulated angiogenesis in this model at 10 µM concentration (Wang et al., 2012). Another key model for *in vivo* angiogenesis is the matrigel plug assay where a matrigel bolus containing the polyphenol in injected into an *in vivo* incubation site, e.g. the hind limb of an animal, and the angiogenesis quantified typically by haemoglobin content at the end of the study. Inclusion of three polyphenols found in hops at 10 µM limited VEGF induced haemoglobin build-up in the matrigel plug (Negrão et al., 2010). Delphinidin, kaempferol, quercetin, apigenin, and luteolin (Lamy et al., 2006, Luo et al., 2009, Pratheeshkumar et al., 2012, Lamy et al., 2008) were all able to inhibit vascularisation of the matrigel plug, with apigenin demonstrating around 45 % inhibition at a 25 µM concentration (Lamy et al., 2008). Following a separate experiment where a VEGF and polyphenol loaded pellet into was implanted in the eye the conjugate quercetin-3-glucuronide, but not quercetin-3-sulphate, could significantly inhibit VEGF induced *in vivo* angiogenesis (Donnini et al., 2006) demonstrating the difference in biological activity that can occur with different metabolites of the same polyphenol. In a modified version of this model where cancer cell lines are included into the matrigel plug alongside the polyphenol, tumour size as well as haemoglobin content has been reported to be inhibited by polyphenols. A polyphenol rich cinnamon extract reduced the haemoglobin content of the matrigel plug containing adenocarcinoma cells (Lu et al., 2010). Curcumin was also able to limit the vessel generation, as measured by haemoglobin content, of the same cell line at a 30 µM concentration (Chakraborty et al., 2008).

Kaempferol was also efficient at limiting both tumour size (46 %) and haemoglobin content (39 %) at 20 µM (Luo et al., 2009). Intraperitoneal (I.P.) injections of 50 mg/kg ellagic acid reduced tumour volume following implantation of adenocarcinoma cell line, although the haemoglobin content of the tumour was not measured (Wang et al., 2012).

1.16 Objectives for the thesis

Considering the previous literature described in this chapter there are two key aims for this thesis. 1) to investigate if and how dietary polyphenols could inhibit VEGF induced VEGFR-2 phosphorylation in HUVECs, 2) to determine whether the polyphenols which elicit potent inhibition of VEGF induced VEGFR-2 phosphorylation could, in turn, inhibit VEGF induced atherosclerosis.

To complete these aims a number of objectives were required to be met. The objectives per chapter are outlined below:

- The objective for chapter 2 is to determine the inhibitory capacity of the bioavailable green tea and pomegranate polyphenols on VEGF-induced VEGFR-2 phosphorylation at physiological concentrations, including the microbial metabolites, the urolithins, which are often overlooked.
- The objective for chapter 3 is to determine whether VEGF supplementation via I.P. injection can induce atherosclerosis in a high cholesterol fed ApoE-/- mouse model, and determine the impact of dietary and injected EGCG and ellagic acid in this model.
- The objective for chapter 4 was to explore the ability of polyphenols to inhibit VEGF-induced-VEGFR-2 phosphorylation in a more physiological *in vitro* model and understand the impact serum proteins can have on polyphenol activity.

Chapter 2

In vitro inhibition of VEGF stimulated VEGFR-2 phosphorylation by dietary polyphenols

2.1 Abstract

VEGF signalling stimulates angiogenesis however upregulation of VEGF signalling is also responsible for pathological angiogenesis. Polyphenols can affect VEGF signalling however for the most part research is undertaken at supra-physiological concentrations, using long incubation times, and overlooking the importance of polyphenol metabolites which limit the conclusions which can be drawn.

In this chapter VEGFR-2 phosphorylation was measured in HUVECs after stimulation with VEGF in the presence of a range of concentrations of EGCG, punicalagin or ellagic acid (EA) as well as the ellagic acid microbial metabolites urolithin A, B, C and D and urolithin A and B glucuronide for 5 minutes. The results were used to calculate the concentration required to inhibit 50 % VEGF-induced VEGFR-2 phosphorylation (IC50) for each polyphenol. Experiments were also conducted which determined whether the polyphenols inhibited VEGF-induced VEGFR-2 phosphorylation through actions on the VEGF ligand or the cell.

EGCG, punicalagin and ellagic acid were demonstrated as potent and rapid inhibitors of VEGF-stimulated VEGFR-2 phosphorylation with IC50 values of 96, 49 and 310 nM respectively. Moreover, the main pomegranate microbial metabolites, the urolithins, were also effective inhibitors. The loss of one hydroxyl group from urolithin D, to urolithin C, then urolithin A and finally urolithin B reduced the inhibitory activity (IC50 values were 0.2, 3.7, 54, and 251 µM respectively). The urolithin glucuronides were ineffective inhibitors at the concentrations tested. It was also demonstrated that punicalagin tested elicited their inhibitory effect predominantly by interacting with the VEGF ligand and not the cell.

The data presented in this chapter provides a strong indication that EGCG and EA are able to inhibit VEGF-induced VEGFR-2 phosphorylation at physiologically relevant concentrations via interactions with the VEGF ligand. Metabolism of the urolithin compounds reduces their efficacy at inhibiting VEGF signalling however urolithin D and urolithin C could inhibit VEGF signalling at concentrations close to those found in the plasma. Both EGCG and EA are viable candidates for further investigation into the polyphenol inhibition of pathological angiogenesis.

2.2 Introduction

Chapter 1 outlined the current understanding of the role of VEGF in angiogenesis, of angiogenesis in atherosclerosis, and of VEGF in atherosclerosis, as well as the current understanding of the effect of polyphenols on these processes. Investigations into the effects of polyphenols on VEGF expression and signalling *in vitro,* until recently, have primarily been undertaken at supra-physiological concentrations without taking into account bioavailability or the physiologically relevant polyphenol metabolites. This chapter will focus on the impact of physiologically relevant polyphenols on inhibition of VEGF signalling.

2.2.1 The VEGF protein

As described in the general introduction VEGF is the primary growth factor responsible for angiogenesis (Ferrara, 2009) through binding to a family of tyrosine kinase receptors known as the VEGF receptors, aided by co-receptors and extracellular complexes such as heparin (Ferrara et al., 2003). There are multi isoforms of VEGF formed through alternative splicing (Krilleke et al., 2009) or proteolytic processing (Ferrara, 2010) however this chapter will focus on the $VEGFA_{165}$ (or $VEGFA_{164}$ in mice) as it is the predominant isoform responsible for VEGF signalling and will simply be referred to as VEGF. VEGF phosphorylation of the VEGFR-2 dimer pair leading to angiogenesis associated signalling. The focus of this chapter is on the activities of VEGFR-2 homodimer receptor pair as it is the primary VEGF receptor responsible for the angiogenesis and neoangiogenesis in the body, including pathological angiogenesis (Goodlad et al., 2006, Doi et al., 2012) as well as being the receptor associated with the response of endothelial cells to VEGF stimulation *in vitro* (Endo et al., 2003).

2.2.2 The VEGF/VEGFR-2 signalling pathway

Upon VEGF ligand binding the VEGF receptor dimer pair switch from their inactive to active state (Sarabipour et al., 2016). Signal transduction occurs via tyrosine phosphorylation sites located along the cytoplasmic domain with each phosphorylation site stimulating a distinct set of secondary signalling molecules. Tyrosine 1054 and 1059 residues are highly conserved across all 3 VEGF receptors

due to their integral role in the kinase activation loop. Without activation of these phosphorylation sites only 10% of the receptor activation is able to occur (Dougher and Terman, 1999). The tyrosine 951 site is found in the kinase insert domain of the receptor. It contributes to Y1175 phosphorylation (Manni et al., 2014) and stimulates membrane bound c-SRC signalling (Sun et al., 2012), vascular permeability and b-Raf dependent cell proliferation (Matsumoto et al., 2005). Phosphorylation at tyrosine 1175 is known to stimulate the majority of angiogenesis signalling by the VEGFR-2 receptor, emphasised by experiments where a mutation at this site induced embryonic death with a similar phenotype to VEGFR-2 null mice (Sakurai et al., 2005b). Signalling from this residue stimulates a number of pathways including the phosphoinositol-3-kinase (PI3K) pathway (Holmqvist et al., 2004), leading to AKT (Cantley, 2002) and eNOS (Fulton et al., 1999) production for cell survival and vascular permeability respectively, and the Plc-γ pathway leading to altered gene expression and endothelial cell migration (Takahashi et al., 2001). For an overview of the key angiogenesis associated VEGF-induced VEGFR-2 signalling pathways see Figure 1.8.

2.2.3 Bioavailability of green tea and pomegranate polyphenols

The bioavailability of polyphenols is an important consideration when investigating their biological action in relation to health. For the polyphenol to elicit the beneficial effect *in vivo* the polyphenol will need to reach an active concentration at the location where the biological effect occurs. VEGFR-2 is a membrane bound receptor, with the stimulant VEGF found in circulation. Therefore, when discussing the effect of polyphenols on VEGF signalling it is important to relate that to the concentration the polyphenol can reach in plasma.

Investigations into green tea polyphenol bioavailability have provided varying results. In humans a high dose (1600 mg) was reported to produce an EGCG plasma concentration of 7 μM (Ullmann et al., 2003) while a lower dose of 193 mg green tea catechins in tea resulted in 174 nM EGCG maximum concentrations in plasma (C_{max}) although bioavailability of EGCG appears to be improved when given as a supplement (Henning et al., 2004). Interestingly EGCG is the only polyphenol found predominantly unconjugated within human plasma following ingestion (Ullmann et

al., 2003, Chow et al., 2001, Meng et al., 2002, Lee et al., 2002). However, in mice it has been reported that EGCG become rapidly glucuronidated after ingestion (Lambert et al., 2003) although EGCG plasma concentrations of 31-50 nM have been reported (Lambert et al., 2003, Dube et al., 2011a, Lambert et al., 2004). Encapsulation of EGCG in nanoparticles increased the polyphenol bioavailability 1.5-fold due to increased stability under gastric conditions (Dube et al., 2011b).

The predominant ellagitannin in pomegranates, punicalagin, is thought to be very poorly bioavailable although was found in rat plasma at 30 µg/ml following feeding with a 6 % punicalagin supplemented diet (Cerda et al., 2003) but this has not been observed in any other species including numerous human intervention trials (Seeram et al., 2004, Cerda et al., 2004, Seeram et al., 2006). However the polyphenol ellagic acid, which is found in the pomegranate fruit as well as being the product of punicalagin hydrolysis, is frequently detected in human plasma with C_{max} values of 20-100 nM after consumption of pomegranate juice or extracts (Seeram et al., 2004, Seeram et al., 2006, Mertens-Talcott et al., 2006). Furthermore feeding of pure ellagic acid resulted in higher plasma concentrations of up to 660 nM (Hamad et al., 2009). In rats peak plasma ellagic acid concentrations of 16.5 µM have been reported (Hamad et al., 2009) although other reports limit this concentration to around 200 nM (Murugan et al., 2009, Lei et al., 2003). Research into the bioavailability of ellagic acid in mice is limited however one report found it in plasma at 38 nM after a 0.8 mg dose (Seeram et al., 2007). However, it has been suggested that the microbial metabolite products of ellagic acid, the urolithins, reach far greater quantities in the plasma than the parent compounds. The concentration of the urolithins in human plasma was reported to exceed 18 µM (Cerda et al., 2004) and for this reason the urolithins have been suggested as the bioactive compound associated with the health benefits of pomegranate. An overview of the ellagic acid metabolism to the main urolithin compounds can be seen in Figure 2.1

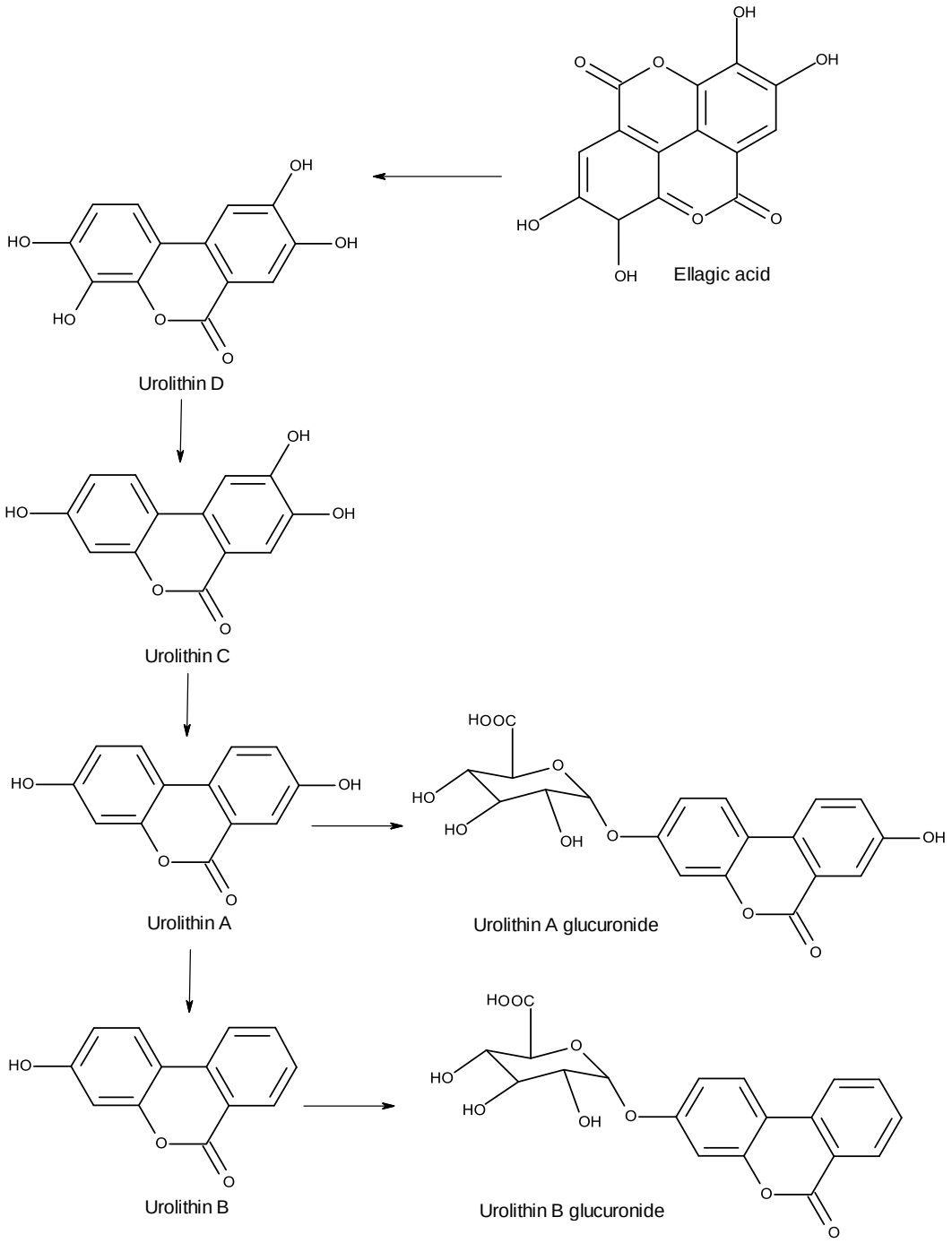


Figure 2.1: Structures and metabolic path production of the microbial metabolites of ellagic acid, the urolithins, and their glucuronides.

2.2.4 Effect of polyphenols on VEGF signalling

Inhibition of VEGFR-2 phosphorylation by a wide variety of polyphenols has been reported in a large range of cell types. EGCG and ellagic acid have both demonstrated inhibition of VEGFR-2 phosphorylation, receptor tyrosine kinase activity and phosphorylation of downstream VEGF signalling partners (Neuhaus et al., 2004, Rodriguez et al., 2006, Shimizu et al., 2010, Tang et al., 2003, Labrecque et al., 2005). EGCG has also been shown to downregulate VEGF gene expression as well as VEGF and VEGFR-2 protein expression. However, these observations were made at supra-physiological concentrations with effective treatments typically ranging from 10 - 50 µM (Shimizu et al., 2010, Lee et al., 2004, Labrecque et al., 2005). Ellagic acid has demonstrated VEGF inhibitory properties at sub 10 µM concentrations. However, long incubation times were used in these studies may have led to polyphenol and VEGF instability over time (Wang et al., 2012). Recently, both EGCG and ellagic acid were demonstrated to have strong effects at nanomolar concentrations on VEGFR-2 phosphorylation in HUVECs after only 5 minutes of treatment (Moyle et al., 2015, Cerezo et al., 2015). In an earlier study *in silico* modelling predicted ellagic acid may elicit its VEGF signalling inhibition by binding the ATP binding region of VEGFR-2 (Wang et al., 2012). However, Cerezo et al. demonstrated a correlation between inhibition of rapid VEGF-stimulated VEGFR-2 phosphorylation and *in silico* predicted affinity of the polyphenols for the receptor binding region of the VEGF ligand. This suggests that VEGF signalling may be affected through polyphenol interactions with the VEGF ligand itself (Cerezo et al., 2015).

Studies into the effect of polyphenol metabolites on VEGF signalling are limited. Metabolism of polyphenols occurs in a number of ways. The phase-II metabolites, quercetin-3-glucuronide and quercetin-3-sulphate, had opposing inhibitory and stimulatory activities on the VEGF signalling pathway (Donnini et al., 2006). The effects of the microbial metabolites are also an important to consider, especially as the inter-individual differences in microbiota may account for the variability in results or effects. For example, the anti-proliferative effects of the urolithins observed in a number of cancer cells lines was significantly reduced through

glucuronidation (González-Sarrías et al., 2014) demonstrating the importance of understanding the effects of metabolism and not just the polyphenols found in the diet. The effect of any microbial metabolites on VEGF signalling has yet to be investigated however it is well documented that research into the bioactivity of metabolites is necessary but currently lacking (Selma et al., 2009, Del Rio et al., 2010).

2.2.5 Objectives

The main objectives of this chapter are: 1) to identify polyphenols which demonstrate potent inhibition of VEGF-induced VEGFR-2 phosphorylation in HUVECs; 2) to determine whether the inhibition of VEGF-induced VEGFR-2 phosphorylation could be achieved at a biologically relevant concentrations; 3) to identify a possible mode of action for polyphenols which elicit potent inhibition of VEGF-induced VEGFR-2 in HUVECs; and 4) to determine whether metabolism can alter the efficacy of VEGF signalling inhibition.

2.3 Material and methods

2.3.1 Subculture of HUVECs

All HUVECs subculture and growth was undertaken in EGM-2 full media which is the EBM-2 basal media (CC-3156, Lonza Group Ltd.) with all growth aliquots added (CC-4176, Lonza Group Ltd.). The EGM-2 media will henceforth be known as 'full media'. HUVECs aliquots were thawed in a water bath at 37°C. The aliquot was pipetted into warmed full media and thoroughly mixed using 10ml stripettes after which, the cell suspension was added drop by drop to the centre of a warmed T75 or T175 flask (Sarstedt Ltd.) to a density of 3500 cells per $cm^{2.}$ The wells were incubated at 37 °C, 5 % CO_2 in a Heracell 150i incubator (Thermo Scientific) until reaching 80-90 % confluency with the media changed every two days. The cells were then removed from the flask by adding trypsin, incubating at 37 °C, 5 % CO_2 for 0.5-1 minute, tapping the sides of the flask to dislodge any stuck cells, and washing the flask surface with warmed full media. The trypsinised cell suspension was moved to a centrifuge tube. The cells were then spun at 250 x g for 5 minutes and the supernatant was carefully removed. The cells were resuspended in 1 ml of cryo-SFM freezing media (Promocell) and counted using a Reichert bright-line haemocytometer (Sigma Aldrich) under a 10 x magnification on an Olympus CK2 inverted microscope, after which the total cell density was calculated. The remaining cell solution was diluted to the correct cell density as required for its planned use in Cryo-SFM, aliquoted into cryovials, and gently frozen in a Mr. Frosty cryocontainer (Nalgene, Thermo Fisher Scientific) filled with isopropanol at -80°C. After 24 hours the cells were transferred to liquid nitrogen for long term storage.

2.3.2 Culture of cells in 6 well plates for treatment

HUVECs were thawed in a water bath at 37°C. The aliquot was pipetted into warmed full media and thoroughly mixed using 10ml stripettes, after which 2 ml of the cell suspension was added drop by drop to the centre of each well of two warmed 6 well plates (Greiner BioOne Ltd) to a density of 3500 cells per cm^2. The plates were incubated at 37°C, 5% CO_2 in a Heracell 150i incubator (Thermo Scientific) and media was changed every 2 days until 100% confluency was achieved.

2.3.3. Determining the IC50 of a polyphenol treatment on VEGFR-2 phosphorylation in HUVECs

EBM-2 basal endothelial medium lacking all growth components and FCS (CC-3156, Lonza Group Ltd.) was supplemented with Gentamycin/Amphotericin and was stored at 4°C for up to one month. From here on in this media will be referred to as simply 'basal media'. Stocks of polyphenols were diluted in DMSO (Sigma Aldrich) to a concentration of 1000 x higher than required in the cell treatments and stored at -20 °C until required. Stocks of recombinant human $VEGF_{165}$ (300-035S, Reliatech) were made from 20 µg lyophilised VEGF reconstituted in phosphate buffered saline (PBS) supplemented with 0.2% bovine serum albumin (BSA) to a concentration of 50 µg/ml. The aliquot of VEGF was defrosted on ice and added to the relevant treatments at a concentration of 1:2000 to give a final VEGF concentration of 25 ng/ml. The polyphenol stocks were added to the appropriate treatments at a 1:1000 ratio and allowed to mix with the VEGF for 5 minutes at room temperature. DMSO was added as a vehicle control to the treatments without polyphenols. Confluent HUVECs grown in 6 well plates (Greiner BioOne Ltd.) were washed twice with 2 ml PBS warmed to 37 °C. After the 5 minute pre-mixing of the VEGF and polyphenol the treatments were added to the appropriate wells and incubated at 37 °C, 5% CO_2 for 5 minutes. The cells were then washed with 2 ml ice cold PBS twice after which 50 µl cell lysis buffer supplemented with 1mM Phenylmethanesulfonyl fluoride solution (PMSF) according to manufacturer's instructions (#9803, Cell Signalling Technology, Inc). Plates were kept at 4 °C for a minimum of 15 minutes after which cells were scraped (541070, Greiner BioOne Ltd.) and the lysates collected. Each lysate was vortexed for 20 seconds, three times at maximum speed with breaks on ice between each vortex. The lysates were then centrifuged for 10 minutes at 13,000 x g at 4 °C and the supernatant was collected in a separate tube. A 5 µl aliquot of each sample was taken prior to freezing to use to determine the total protein concentration in each sample. The sample and 5 µl aliquot were frozen and stored at -80 °C until analysis could be performed. An overview of the workflow for this assay can be seen in Figure 2.2.

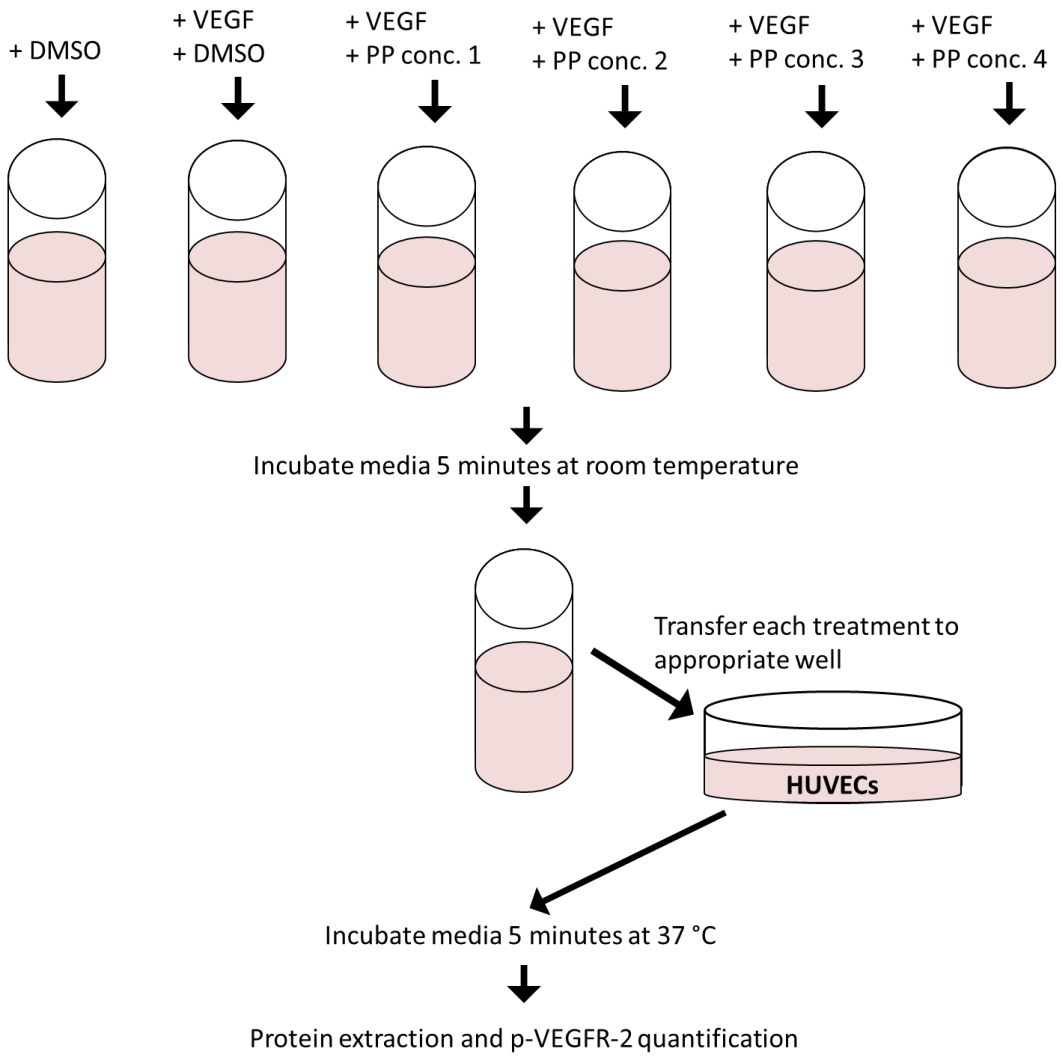


Figure 2.2: An overview of the work flow required to determine an IC50 value described in section 2.3.3

2.3.4 Determining where the polyphenol acts to elicit any observed inhibitory effects.

As before stocks of polyphenols and recombinant human $VEGF_{165}$ (300-035S, Reliatech) were made as described earlier in section 2.3.3. The concentration of polyphenols used in these experiments was determined as a strongly inhibitory concentration based upon the data from the IC50 experiments. One treatment was designed to treat the cells first with the polyphenol, followed by an independent VEGF treatment (named 'independent' treatments). The cells requiring independent treatment were washed twice with warmed PBS after which the appropriate wells were treated with basal media supplemented with the

polyphenol to the required concentration at 1:1000 dilution and the cells were incubated for 5 minutes at 37 °C, 5% CO_2. VEGF was added to the treatment media requiring VEGF supplementation in a 1:2000 ratio to concentration of 25 ng/ml. One volume of VEGF only supplemented media was to be used for the independent treatment after the 5 minute polyphenol only treatment was complete. Another volume of VEGF supplemented media was mixed with the polyphenol for 5 minutes to create a treatment where the VEGF and polyphenol are added to the cells in a pre-mixed manner ('combined' treatment). DMSO was added as a vehicle control to the treatments without polyphenols. After the polyphenol only independent 5 minute treatment was complete all cells were washed with warmed PBS twice and 2 ml of either the VEGF only or VEGF and polyphenol treatments were added to the 'independent' and 'combined' treated cells respectively and incubated at 37°C, 5% CO_2 for 5 minutes. Following this final incubation all wells were washed with 2 ml ice cold PBS twice. Cells were lysed with 50 µl cell lysis buffer supplemented with 1mM PMSF (9803, Cell Signalling Technology Inc., New England Biolabs), scraped, and the lysates collected. Each lysate was vortexed for 20 seconds three times at maximum speed with breaks on ice between each vortex. The lysates were then centrifuged for 10 minutes at 13,000 x g at 4 °C and the supernatant was collected in a separate tube. A 5 µl aliquot of each sample was taken prior to freezing to use to determine the total protein concentration in each sample. An overview of the workflow for this assay Is shown in Figure 2.3.

2.3.5 Quantification of the absolute protein concentration of cell lysates

To quantify the total protein a sample the bicinchoninic acid (BCA) assay was used. For each BCA assay a BSA calibration curve ranging from 0 mg/ml to 1 mg/ml was set up. 55 µl of sodium phosphate buffer (NaPi) buffer was added to each of the 5 µl experimental samples. 25 µl of each sample were transferred to two wells of a 96 well plate in duplicate. A solution of Bicinchoninic acid and copper II sulphate in a ratio of 50:1 v:v. was made and 200 µl of this was added to each of the wells containing sample. The plate was left at 37 °C for 30 minutes to complete the reaction after which the plate was immediately read using a FluorStar OPTIMA spectrophotometer (BMG Labtech) at 450nm.

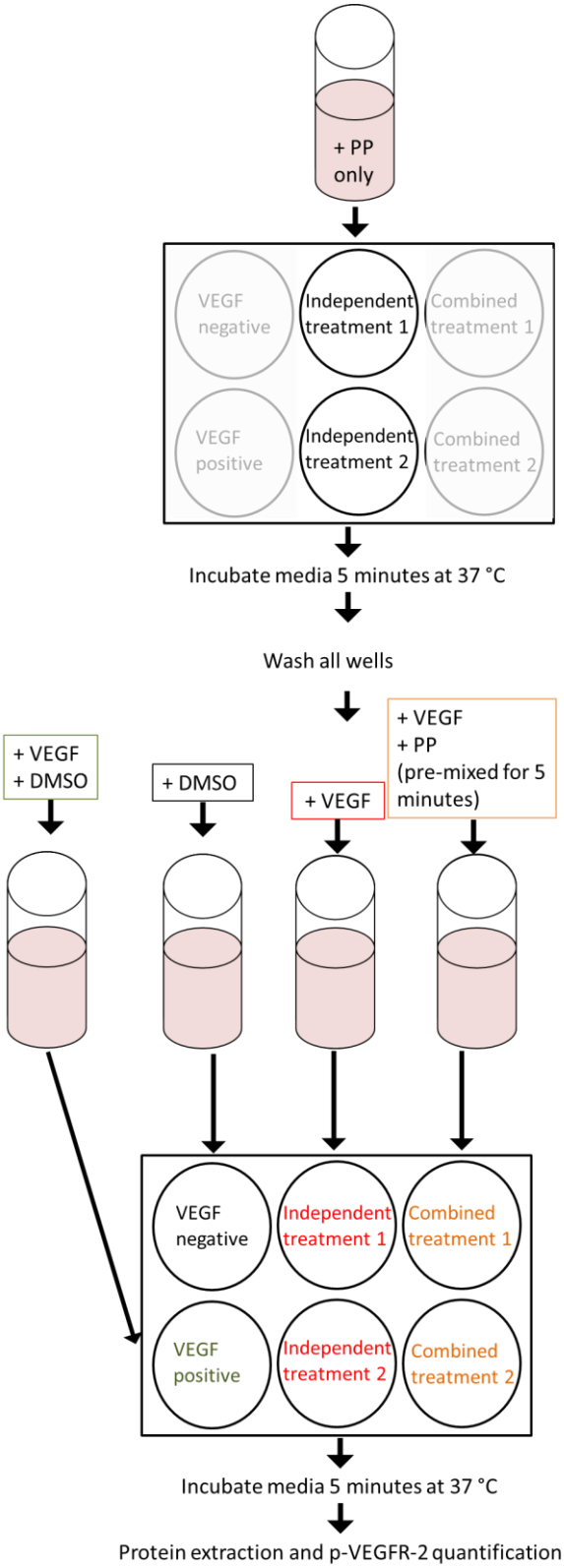


Figure 2.3: An overview of the work flow required to determine where the polyphenol elicits its inhibitory activity as described in section 2.3.4

2.3.6 Determining the amount of VEGF receptor phosphorylation in HUVECs following cell treatments

An enzyme linked immunosorbent assay (ELISA) kit was used (7335, New England Biolabs) to determine the amount of tyrosine 1175 (Y1175) VEGFR-2 phosphorylation in the cell lysates. ELISAs were run according to the manufacturer's instructions. Briefly, the samples were diluted in MilliQ water to the same total protein concentration in a volume of 110 µl. The samples were diluted 1:1 with the sample diluent provided to give a total volume of 220 µl. 100 µl of each sample was added per well of a mouse anti-VEGFR-2 antibody pre-coated ELISA plate. The plates were sealed and stored overnight at 4 °C. The following morning the wells washed four times with 1x wash buffer. 100 µl of the rabbit anti-phosphorylated Y1175-VEGFR-2 primary antibody was added to each well and the plates were sealed and incubated at 37 °C for 1 h. Following this the plates washed a further four times with 1x wash buffer. 100 µl of the anti-rabbit HRP-linked secondary antibody was added to each well, the plates were sealed and incubated at 37 °C for 30 minutes after which the plates were again washed four times with 1x wash buffer. 100 µl TMB substrate solution was added to each well, the plates were sealed and incubated for 10 minutes at 37 °C. Following this incubation 100 µl STOP solution was added directly to the TMB substrate and the plates were read immediately at 450nm-540 nm on a FluorStar OPTIMA spectrophotometer (BMG Labtech). The percentage VEGFR-2 phosphorylation in comparison to a VEGF stimulated sample was calculated.

2.3.7 Statistical analysis

The VEGFR-2 phosphorylation percentages of each treatment group in comparison to the VEGF positive control were plotted into both a bar chart and line graphs using the GraphPad Prism software. Any statistical difference between the groups was determined by ANOVA with a Tukey post-hoc test comparing all sample groups to one another. The IC50 was calculated following logarithmic transformation and non-linear regression analysis of the data. The IC50 is the half way point between the bottom and top plateaus of the curve of the transformed data

2.4 Results

2.4.1 The green tea polyphenol epigallocatechin gallate effectively inhibits VEGFR-2 signalling in HUVECs

As shown previously (Moyle et al., 2015) treatment of HUVECs with VEGF that has been pre-mixed with EGCG at 1 µM for 5 minutes prior to treatment strongly inhibits VEGFR-2 phosphorylation. This experiment was replicated to confirm the same observation could be made. EGCG at both a 0.1 µM and 1µM concentration significantly inhibited the VEGF-induced VEGFR-2 phosphorylation after 5 minute treatment with EBM-2 media containing pre-mixed VEGF (25 ng/ml) and EGCG. There was also a significant decrease in VEGFR-2 phosphorylation when treating HUVECs with VEGF pre-mixed with the higher EGCG concentration of 1 µM in comparison to the 0.1 µM concentration, suggesting a higher concentration of EGCG is able to more strongly reduce VEGF stimulated VEGFR-2 phosphorylation (Figure 2.4). Following non-linear regression analysis of the transformed data an IC50 value of 96 nM (95% CI 91.1 nM to 102 nM) was calculated.

2.4.2 The pomegranate polyphenol punicalagin effectively inhibits VEGFR-2 signalling in HUVECs

The primary ellagitannin in pomegranates, punicalagin was pre-mixed with VEGF (25 ng/ml) in EBM-2 basal media at varying concentrations. 0.01µM did not show a decrease in VEGFR-2 phosphorylation in comparison to VEGF only stimulated cells. All other concentrations tested resulted in a significant reduction in VEGFR-2 phosphorylation, with increasing concentrations of punicalagin corresponding to a greater decrease in VEGFR-2 phosphorylation in comparison to a VEGF only stimulated control (Figure 2.5). Following transformation of the data to a logarithmic scale and undertaking of a non-linear regression analysis the IC50 of punicalagin was estimated as 49 nM (95% CI 43.0 µM to 57.3 µM).

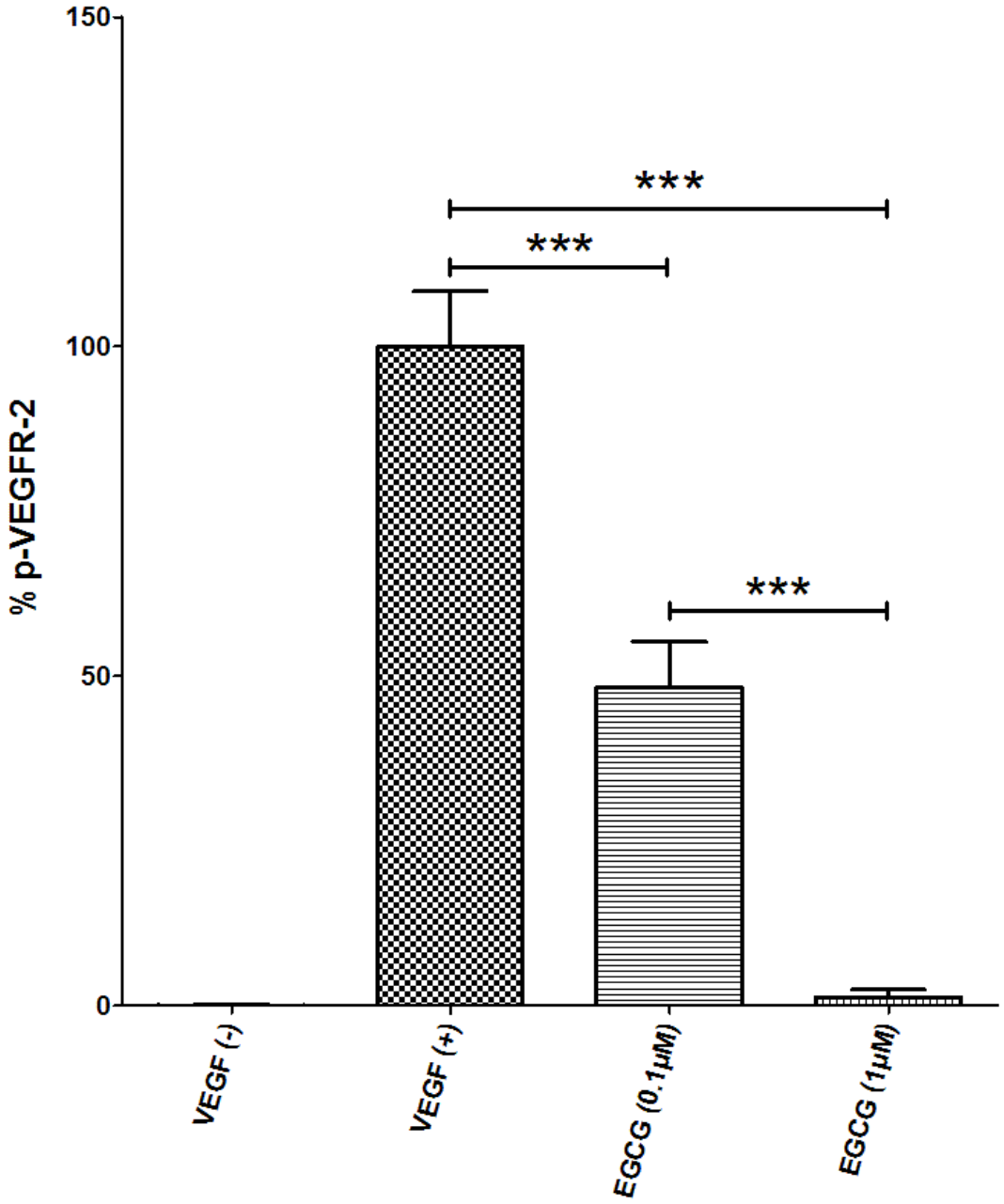


Figure 2.4:.EGCG inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus either 0.1 or 1 µM EGCG and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 4.

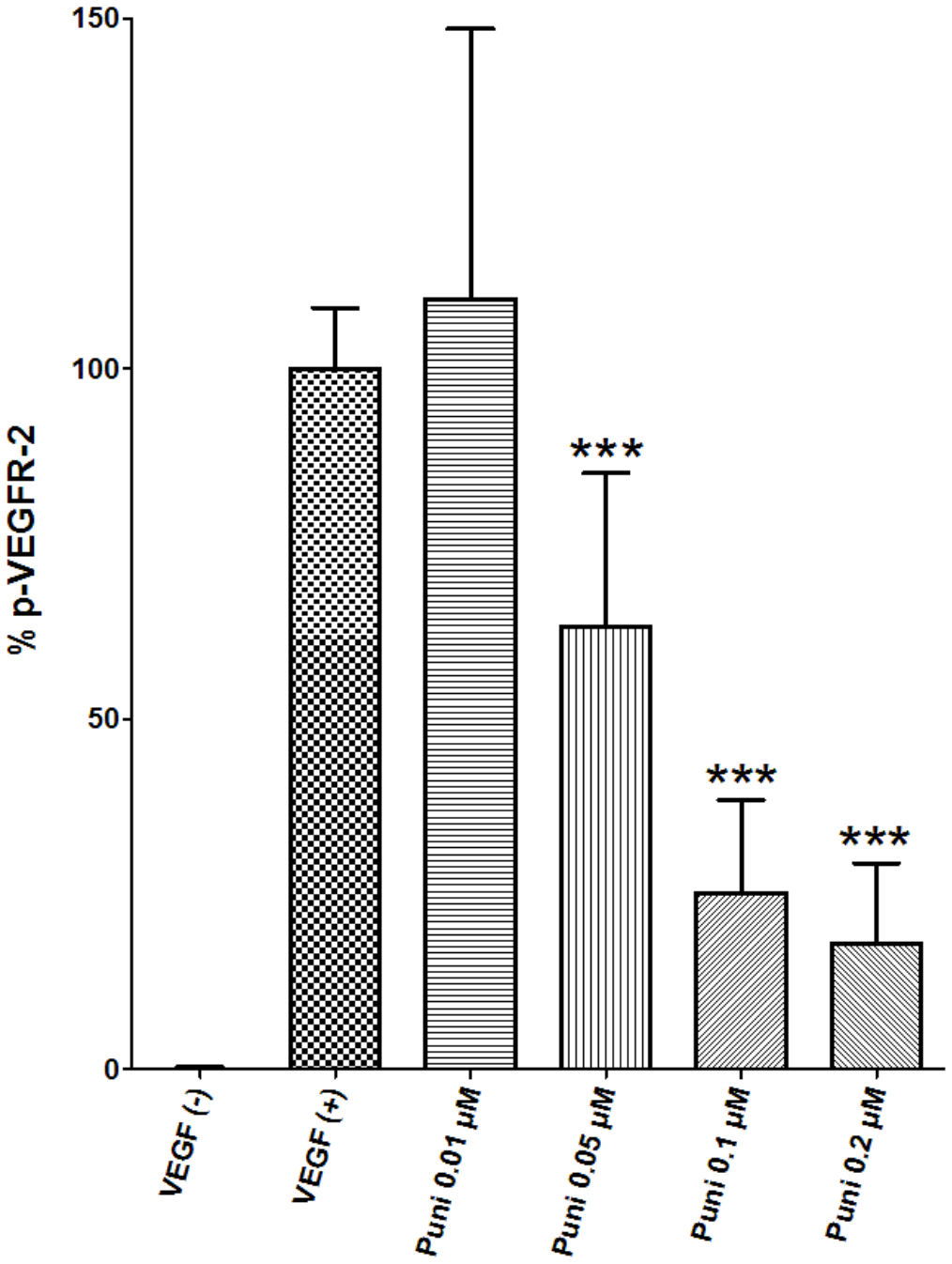


Figure 2.5: Punicalagin inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of punicalagin (range: 0.01-0.2µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. *** $p \leq 0.001$ as compared to the VEGF stimulated control; .n = 5.

2.4.3 The pomegranate polyphenol ellagic acid effectively inhibits VEGFR-2 signalling in HUVECs

Ellagic acid is a compound found both in pomegranate products and is also a primary breakdown product of punicalagin. The IC50 of EA on VEGFR-2 phosphorylation in HUVECs had previously been estimated to be 0.230 (95% CI 0.214-0.247) (Cerezo et al., 2015). Figure 2.6 shows the VEGF-induced VEGFR-2 phosphorylation achieved in the presence of increasing concentrations of ellagic acid in this experiment. A significantly reduced p-VEGFR-2 percentage is observed following treatment with VEGF pre-mixed with EA at 0.15 µM or higher and as the concentration of the EA increases the VEGF stimulated VEGFR-2 phosphorylation decreases. Following transformation of the data to a logarithmic scale and undertaking of a non-linear regression analysis the relative IC50 of ellagic acid from this data was determined as 0.31 µM (95% CI 0.1614 µM to 0.5950 µM).

2.4.4 The inhibition capacity of the pomegranate derived microbial metabolites on p-VEGFR-2

Following ingestion of pomegranate the predominant metabolites, formed by microbial metabolism, are the urolithin compounds. The VEGFR-2 phosphorylation inhibition capabilities of these metabolites, determined by IC50 where possible, were tested using the same *in vitro* protocol as for EGCG, punicalagin, and EA. Urolithin D, the initial tetra-hydroxy microbial metabolite, demonstrated a potent inhibitory effect on p-VEGFR-2 at all concentrations tested. Nearly complete inhibition of VEGFR-2 phosphorylation was seen following pre-mixing of the VEGF with urolithin D at a concentration of 3 µM (Figure 2.7). Following transformation of the data to a logarithmic scale and undertaking of a non-linear regression analysis the relative IC50 of urolithin D was determined as 0.20 µM (95% CI 0.106 µM to 0.357 µM).

Urolithin C is a tri-hydroxy compounds and follows urolithin D in the metabolism pathway. Urolithin C was tested at four different concentrations (2, 4, 8, and 16 µM) to determine the IC50 for VEGFR-2 phosphorylation inhibition in the assay (Figure 2.8). All tested concentrations significantly reduced the percentage VEGFR-2

phosphorylation at Y1175 in comparison to a VEGF stimulated control with near complete inhibition occurring following co-treatment with both 8 µM urolithin C (3 % p-VEGFR-2) and 16 µM urolithin C (0.6 % p-VEGFR-2). Following transformation of the data to a logarithmic scale and undertaking of a non-linear regression analysis the relative IC50 of urolithin C was determined as 3.70 µM (95% CI 3.40 µM to 4.01 µM).

Urolithin A is a di-hydroxy compound which appears after urolithin C and D in the plasma after consumption of pomegranates. Using the *in vitro* assay to investigate the effect of urolithin A on VEGF stimulated VEGFR-2 phosphorylation it was shown that urolithin A had a reduced ability to inhibit this signalling in comparison to the previously tested urolithins. Significant inhibition of VEGF-induced VEGFR-2 phosphorylation was observed following pre-mixing of the VEGF with 40 and 60 µM urolithin A. Complete inhibition of p-VEGFR-2 could not be achieved at any of the concentration tested (Figure 2.9). Following transformation of the data to a logarithmic scale and undertaking of a non-linear regression analysis the relative IC50 of urolithin A was determined as 54.0 µM (95% CI 45.6 µM to 63.8 µM).

Urolithin B is a mono-hydroxy compound and, along with urolithin A, is a terminal urolithin produced through metabolism of pomegranate polyphenols. Urolithin B was tested at a range of concentrations (20, 60, 90, and 120 µM) up to a level that exceeded the expected plasma concentration following high amounts of pomegranate ingestion. 20 and 60 µM did not significantly inhibit VEGF induced VEGFR-2 phosphorylation while pre-mixing the VEGF with 90 and 120 µM urolithin B resulted in a small but significant decrease in VEGFR-2 phosphorylation (Figure 2.10). An IC50 value is difficult to accurately interpret from the results as no treatment with urolithin B was able to reduce the VEGFR-2 phosphorylation to below 50 %. However, following transformation and non-linear regression analysis an IC50 of 251 µM was determined with a wide 95 % confidence interval (131 to 484 µM).

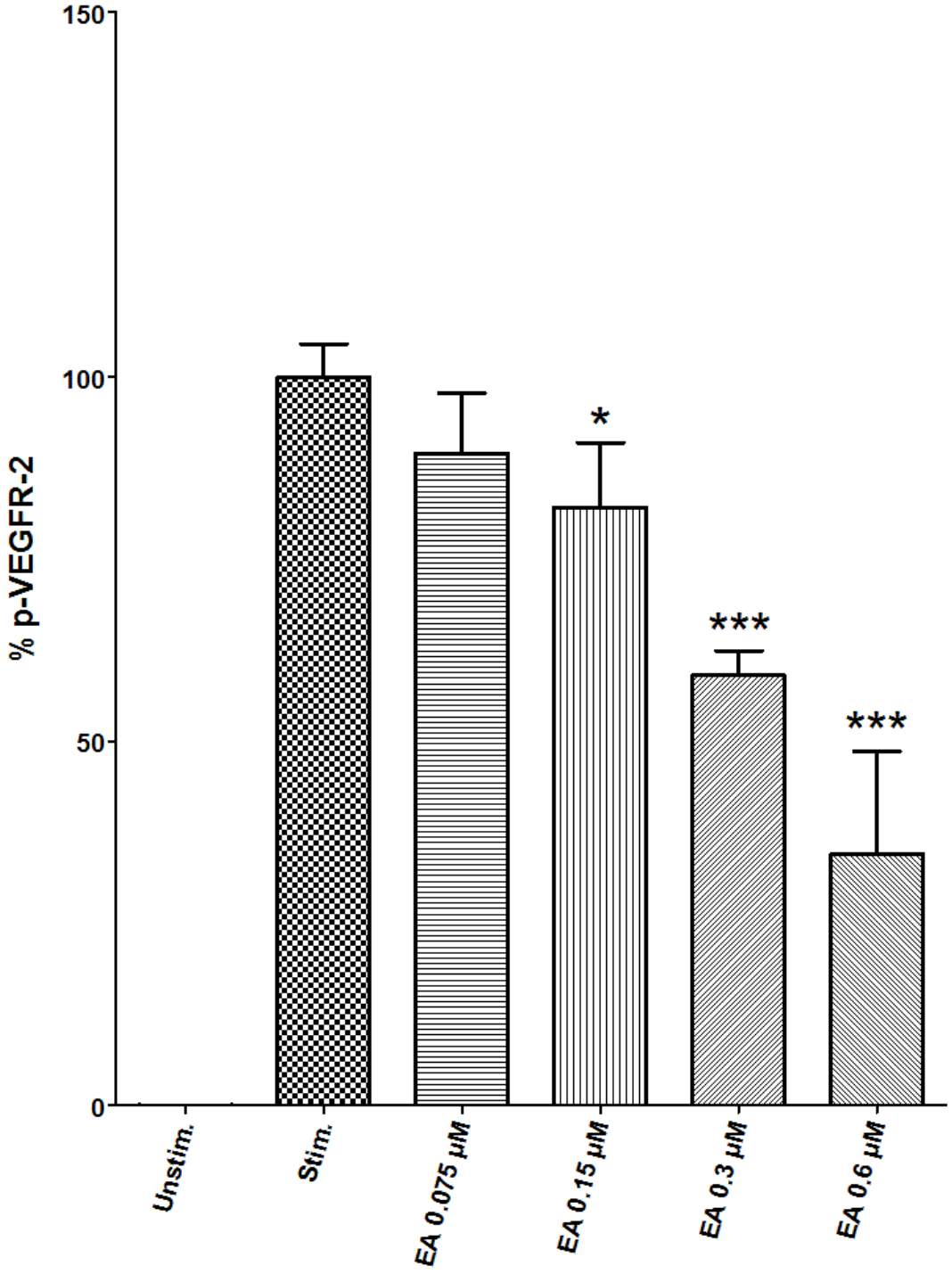


Figure 2.6: Ellagic acid inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of EA (range: 0.075-0.6 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. * $p \leq 0.05$, *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 4.

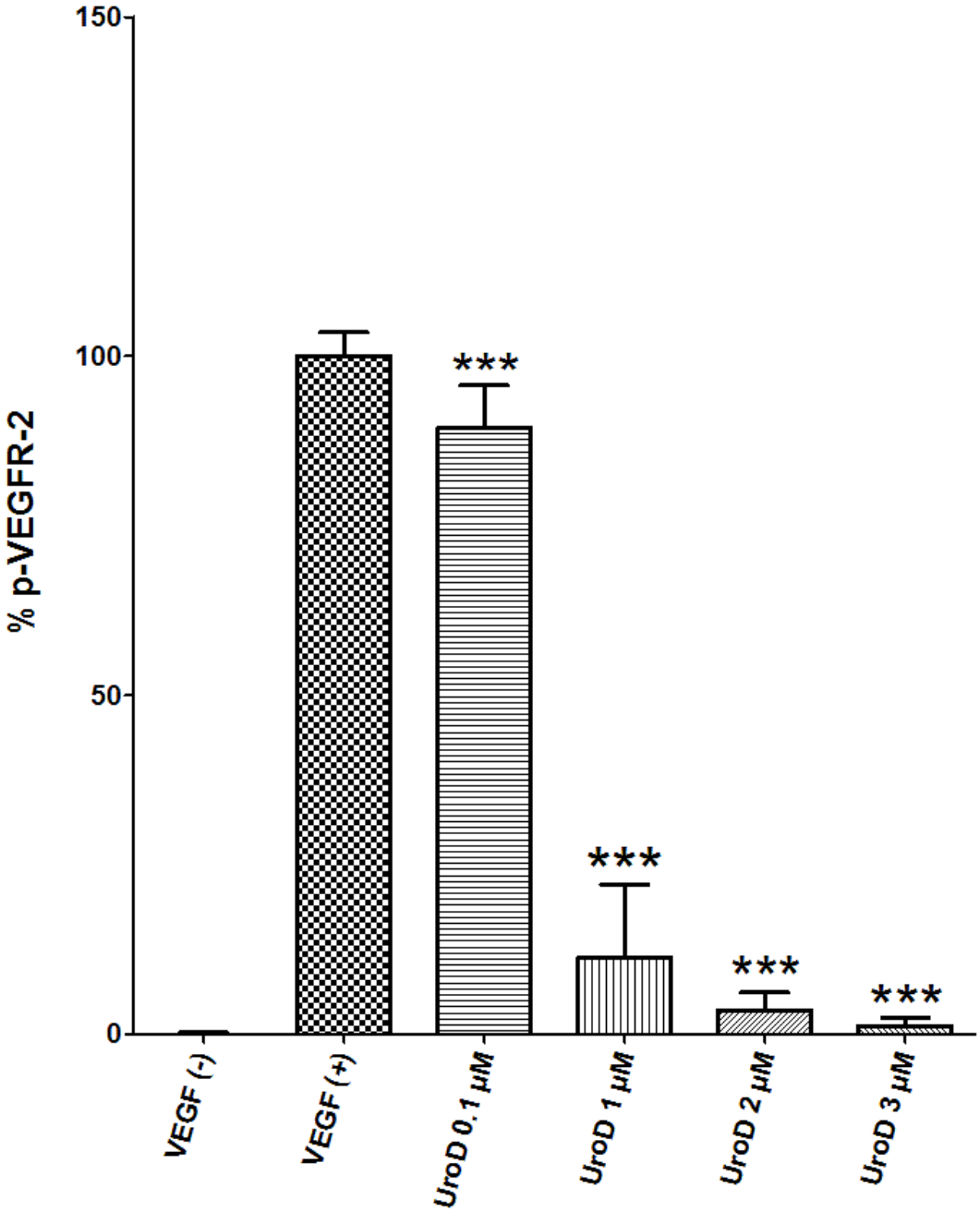


Figure 2.7: Urolithin D inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin D (range: 0.1-3 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. * $p \leq 0.05$, *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 6.

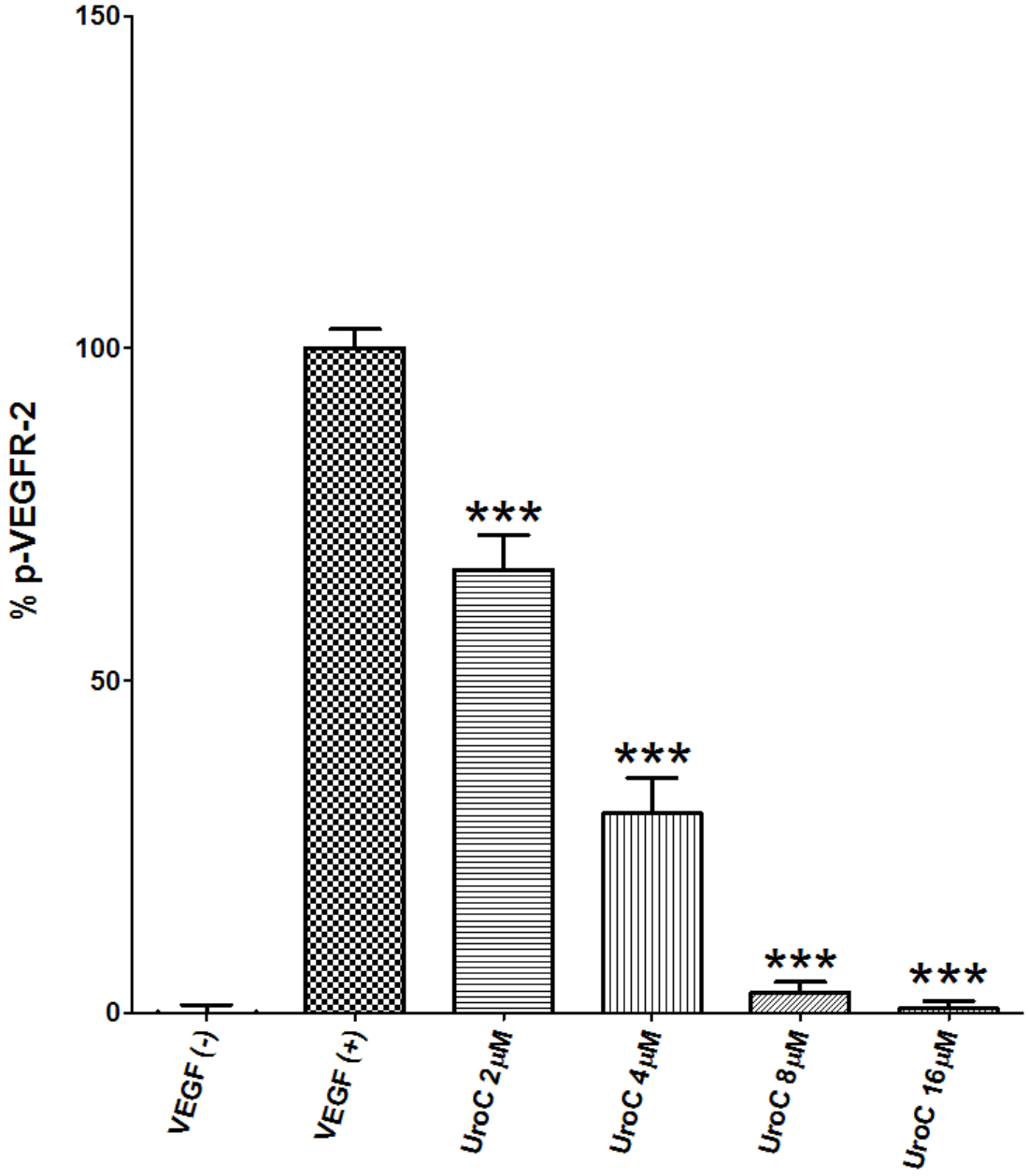


Figure 2.8: Urolithin C inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin C (range: 2-16 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. * $p \leq 0.05$, *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 6.

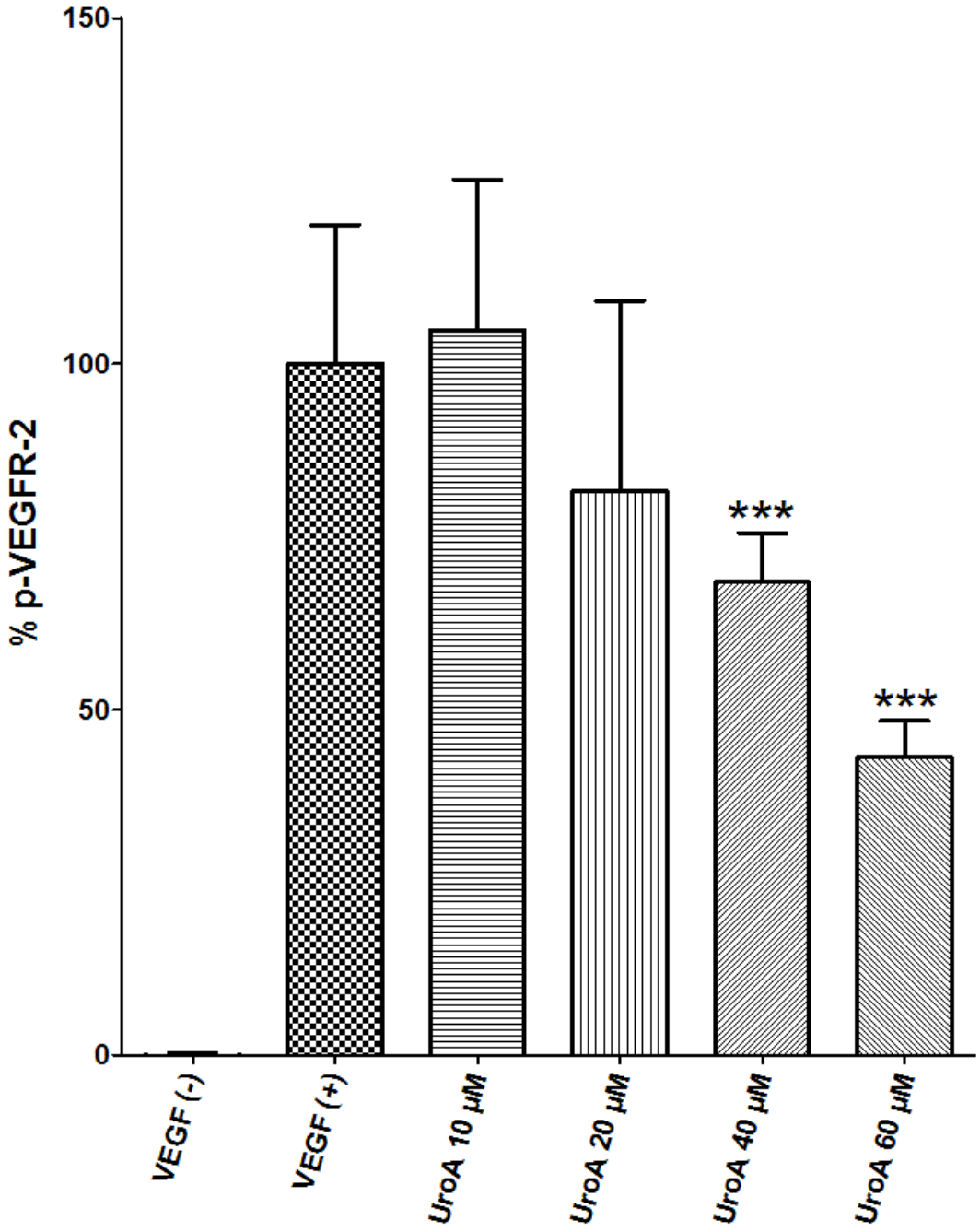


Figure 2.9: Urolithin A inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin A (range: 10-60 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 6.

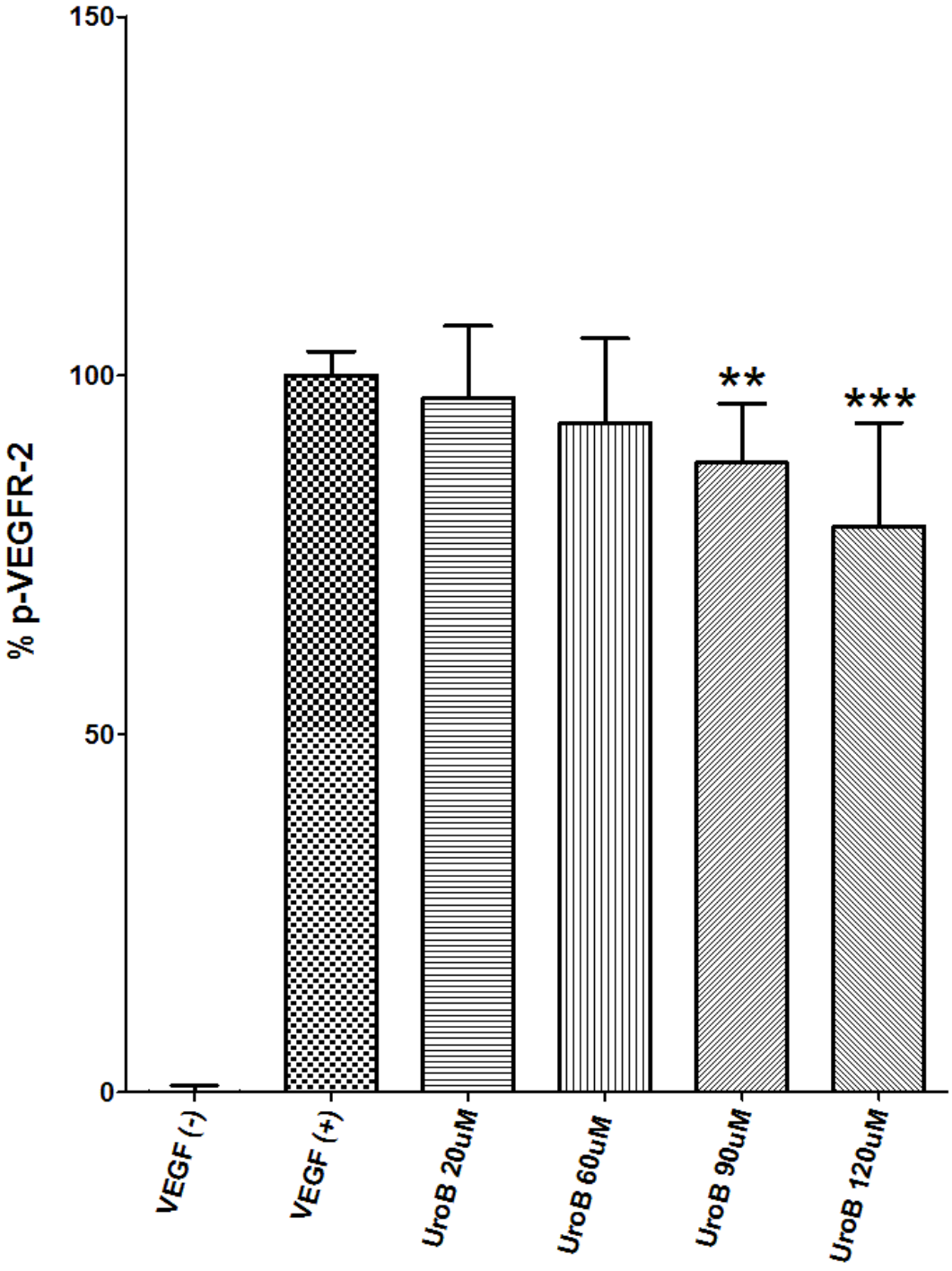


Figure 2.10: Urolithin B inhibition of VEGF induced VEGFR-2 phosphorylation

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin B (range: 20-120 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control. ** $p \leq 0.01$, *** $p \leq 0.001$ as compared to the VEGF stimulated control; n = 6.

2.4.5 Conjugation of metabolites causes loss of inhibitory capacity

In vivo urolithin D and C can be found in the plasma but are relatively rapidly converted to urolithin A and B which are the terminal urolithins. Urolithin A-glucuronide and urolithin B-glucuronide are produced by the liver through conjugation and are found in comparatively high quantities in the plasma. Urolithin A and B glucuronide were investigated for their VEGFR-2 phosphorylation inhibition capacity using the same model as for the unconjugated polyphenols. Structures of urolithin A-glucuronide and urolithin B-glucuronide can be seen in Figure 2.1.

Following pre-mixing of VEGF with urolithin A-glucuronide or urolithin B-glucuronide at a wide range of concentrations (10-150 µM) there was no significant inhibition of VEGF-induced VEGFR-2 phosphorylation at any concentrations tested (Figure 2.11 and Figure 2.12) for either compound. Due to a complete lack in inhibitory capacity in this model an IC50 could not be determined for comparison.

2.4.6 Green tea and pomegranate polyphenols elicit their anti-VEGFR-2 phosphorylation activity by acting primarily on the VEGF ligand.

It has previously been reported that EGCG is capable of inhibiting VEGFR-2 phosphorylation by interacting with the VEGF ligand (Moyle et al., 2015). To test this, an experiment was conducted where the VEGF and polyphenol are either pre-mixed for 5 minutes prior to treatment, named 'Combined' treatment, or where the cells are treated with the polyphenol only for 5 minutes followed by a second 5 minute VEGF only stimulation, called 'independent' treatment. As expected the 'combined' treatments, where VEGF was pre-mixed with EGCG, punicalagin or EA (1 µM) or urolithin D and C (10 µM) for 5 minutes, resulted in near complete inhibition of VEGF-induced p-VEGFR-2. In the 'independent' treatment which underwent a 5 minute polyphenol only treatment followed by 5 minute VEGF stimulation little to no inhibition of VEGF-induced VEGFR-2 phosphorylation was observed (0-10 %). Interestingly, punicalagin, EA, and urolithin D only treatment of the cell ('independent' treatment) resulted in around 10% VEGFR-2 phosphorylation inhibition without coming into contact with the VEGF ligand. This suggests this small effect was caused by actions on the cells directly (Figure 2.13).

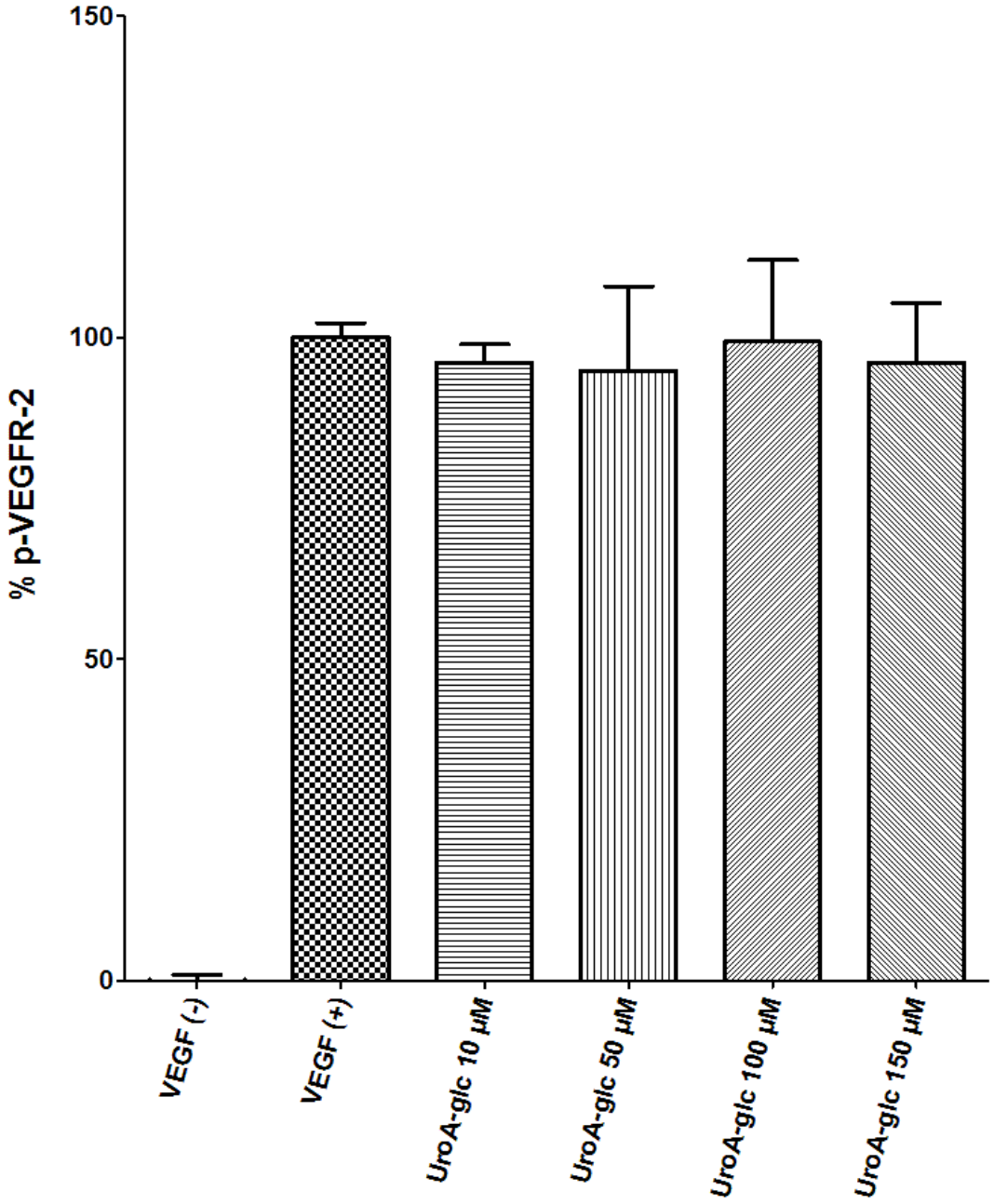


Figure 2.11: VEGF induced VEGFR-2 phosphorylation after pre-mixing with urolithin A glucuronide

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin A - glucuronide (range: 10-150 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control; n = 6.

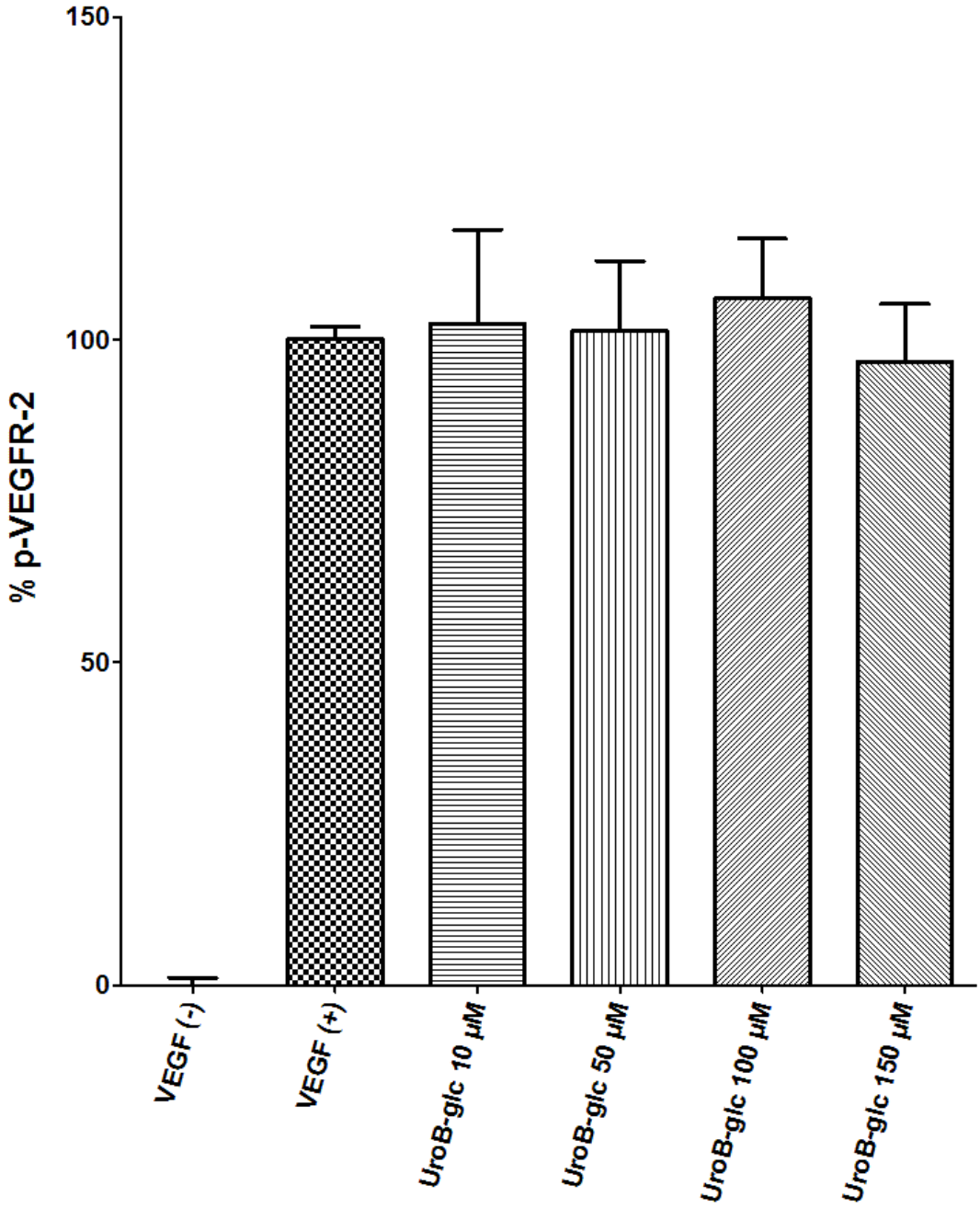


Figure 2.12: VEGF induced VEGFR-2 phosphorylation after pre-mixing with urolithin B glucuronide

EBM-2 media was supplemented with 25 ng/ml VEGF plus varying concentrations of urolithin B - glucuronide (range: 10-150 µM) and allowed to mix for 5 minutes. The supplemented media was then used to stimulate HUVECs for 5 minutes at 37 °C, after which the total protein was extracted and the amount of phosphorylated VEGFR-2 determined. Bars represent means ± standard deviation of % p-VEGFR-2 in comparison to the VEGF only stimulated control; n = 6.

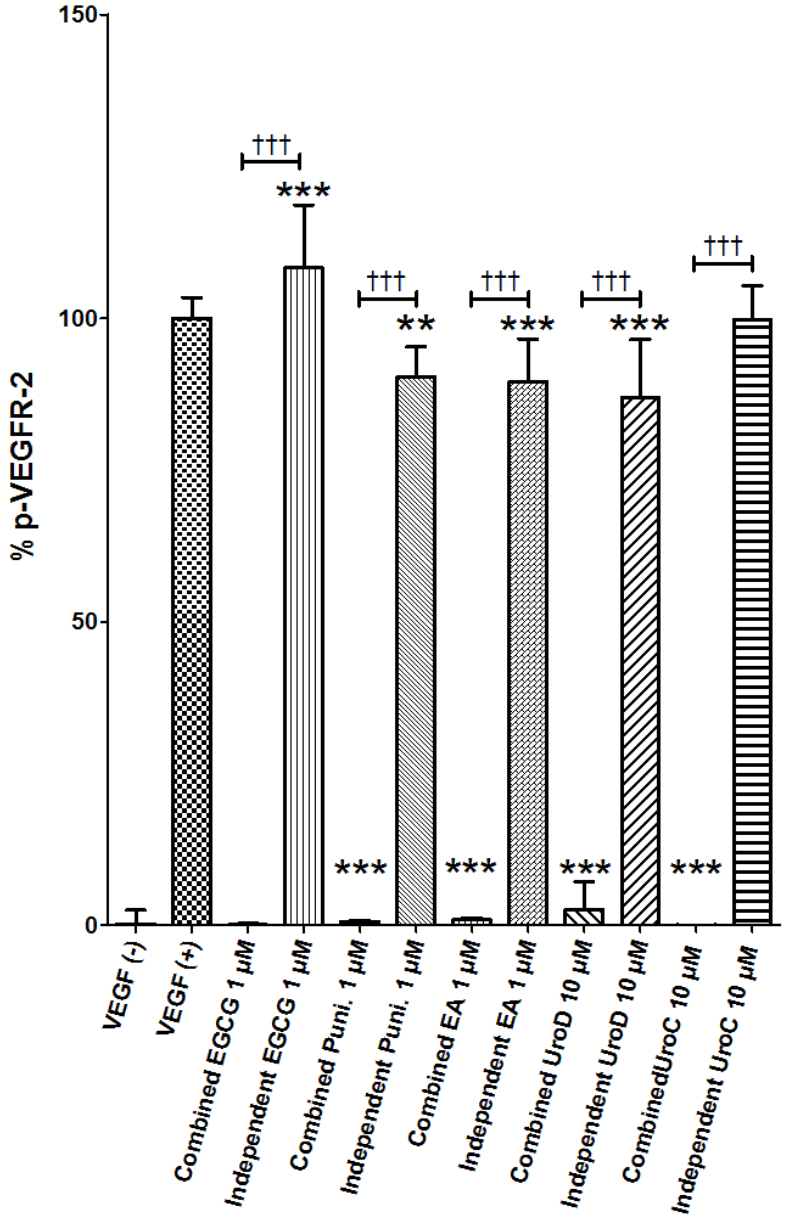


Figure 2.13: The VEGF Induced VEGFR-2 phosphorylation following VEGF and polyphenol combined treatment or independent treatment of HUVECs

All dietary sourced polyphenol with complete or near-complete inhibition of p-VEGFR-2 inhibition at 1 µM were investigated for their mode of action. All urolithin compounds with complete or near-complete inhibition of p-VEGFR-2 at 10 µM were also investigated for the mode of action. VEGF and the polyphenol were either pre-mixed for 5 minutes prior to treatment of cells for 5 minutes (combined) or the polyphenol only in EBM-2 media was added to the cell for 5 minutes (independent) after which the cells were washed and stimulated with VEGF only for 5 minutes. Total protein was extracted and VEGFR-2 phosphorylation determined by ELISA. ** $p \leq 0.01$, *** $p \leq 0.001$ as compared to a VEGF only stimulated sample. ††† $p \leq 0.001$ between independent and combined treatments with the same polyphenol; n = 4.

2.5 Discussion

The data presented in this chapter confirms the previous observation that the green tea polyphenol EGCG and the pomegranate polyphenol ellagic acid are strong inhibitors of VEGF stimulated VEGFR-2. It is also the first time it has been shown that: 1) the predominant pomegranate polyphenol, punicalagin, is a strong inhibitor of VEGF-induced VEGFR-2 phosphorylation; 2) Microbial metabolites of ellagic acid are able to inhibit VEGF-induced VEGFR-2 phosphorylation; 3) all processes of punicalagin metabolism, be it hydrolysis, microbial, or conjugation reduce the potency of inhibition in this model; 4) where tested, the potent inhibition of VEGF-stimulated VEGFR-2 phosphorylation by green tea and pomegranate polyphenols was predominantly through interaction with the VEGF ligand and not the cell.

Inhibition of VEGF signalling has an important role in clinical treatment of conditions such as cancer and wet-related macular degeneration (Massin et al., 2010, Ferrara et al., 2005) and VEGF has been linked to the progression of diseases such as atherosclerosis (Celletti et al., 2001a, Celletti et al., 2001b). However, side-effects from current anti-VEGF treatments are starting to emerge (Kamba and McDonald, 2007) such as increased venous thromboembolic events (Thulliez et al., 2014). Dietary intervention may provide an alternative to such treatments given they can inhibit VEGF-induced VEGFR-2 phosphorylation at biologically relevant concentrations *in vitro* and have been found to be non-toxic in high doses (Isbrucker et al., 2006, Cerdá et al., 2003, Tasaki et al., 2008).

It was previously demonstrated that EGCG and ellagic acid were able to effectively inhibit VEGFR-2 phosphorylation without significantly altering the total VEGFR-2 protein level (Labrecque et al., 2005, Lamy et al., 2002), as well as inhibiting the phosphorylation of VEGF associated downstream signalling partners such as AKT, eNOS and ERK. However, these experiments used supra-physiological concentrations to demonstrate the effects on cell signalling pathways, as well as long incubation times. Treatments with supra-physiological concentrations can provide an indication as to which polyphenols may be more efficient inhibitors of the VEGF signalling pathway but if the concentrations used cannot be achieved in the plasma, the physiological effects are not relevant to an *in vivo* setting. Equally,

polyphenols are known to be unstable under cell culture conditions to a lesser or greater extent (Xiao and Hogger, 2015) Therefore, as they didn't analyse the treatment media it cannot be determined whether the effect is due to the original polyphenol or its breakdown components. Longer incubations of EGCG are also known to make a number of changes to cell media which has the potential to influence the conclusions from the experiment (Tao et al., 2014). Furthermore, phosphorylation is a rapid event (Fearnley et al., 2016) therefore longer incubation times when discussing phosphorylation inhibition may not be the most suitable study design. More recently data has been emerging on the direct effect of a variety of polyphenols to rapidly inhibit VEGF-induced VEGFR-2 phosphorylation. Following a 5 minute pre-mixing of the VEGF with the polyphenol the cells are stimulated for 5 minutes with the mixture and the VEGFR-2 phosphorylation quantified. Given VEGFR-2 phosphorylation peaks at 5-10 minutes post stimulation (Fearnley et al., 2016) this represents the immediate effects of polyphenols on VEGF signalling. In these studies, both EGCG and ellagic acid were shown to be strong inhibitors with IC50 values of 88 nM and 230 nM concentration respectively (Cerezo et al., 2015, Moyle et al., 2015).

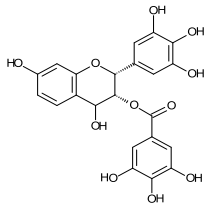
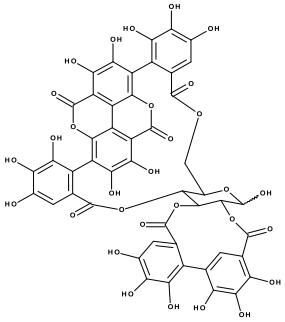
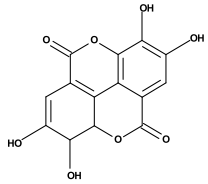
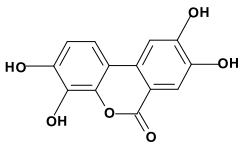
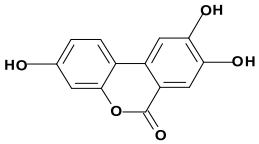
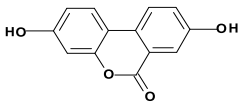
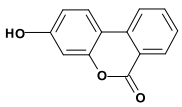
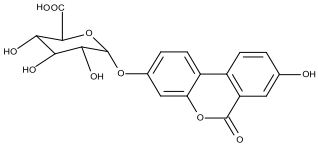
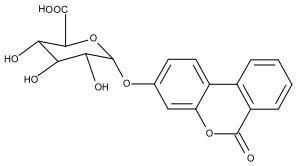
2.5.1 Green tea polyphenol inhibition of VEGF signalling

The data presented in this chapter used the same premixed VEGF and polyphenol 5 minute treatment as described above (Cerezo et al., 2015, Moyle et al., 2015). A summary of the IC50 values reported in this chapter can be seen in Table 2.1. EGCG was able to inhibit 50 % of VEGFR-2 phosphorylation at 96 nM, a similar value to that achieved previously (88 nM) (Cerezo et al., 2015), demonstrating that the HUVECs were an effective, operator independent reporter of VEGF stimulated VEGFR-2 phosphorylation. The potent inhibition of VEGFR-2 phosphorylation achieved with EGCG is at a much lower concentration than previously reported. Lamy et. al. (Lamy et al., 2002) reported near complete inhibition of p-VEGFR-2 following 10 μM EGCG pre-treatment for 24 hours. However, the inhibition observed in the study by Lamy et. al. is not directly comparable due to the long incubation times, the fact that a pan-tyrosine phosphorylation of VEGFR-2 immunoprecipitated protein was used for quantification, and that the EGCG was

only able to elicit its effect via actions on the cell and never came into contact with the VEGF ligand. However, it suggests that EGCG and the degradation products do have a role in sustained inhibition of pan VEGF receptor phosphorylation.

Bioavailability is an important consideration when discussing *in vitro* observations if they are to be considered relevant to an *in vivo* setting, although translational research plays another key role in this conclusion. Henning et. al. compared the plasma concentrations achieved following a similar dose in green tea, black tea and a green tea extract (193.3., 230.8, 213.6 mg respectively). It demonstrated that EGCG was able to reach plasma concentrations of 174, 218, and 326 nM respectively following green tea, black tea and green tea extract intake suggesting that the source of the polyphenol contributed to the plasma concentration achieved (Henning et al., 2004). Other studies have looked at the bioavailability of the pure compound EGCG, at doses ranging from 50-1600 mg (Chow et al., 2001, Ullmann et al., 2003, Henning et al., 2005) with the highest dose being reported to induce around 7 μM, a highly effective concentration for inhibiting VEGF induced VEGFR-2 phosphorylation in the *in vitro* model presented in this chapter. However, without further supplementation to the diet these concentrations are unlikely to appear in human plasma through dietary intake alone. It is also important to note that EGCG is often found predominantly as an aglycone in plasma (Ullmann et al., 2003, Chow et al., 2001, Meng et al., 2002, Lee et al., 2002). Therefore it can be concluded that EGCG has the potential to reach concentrations within the plasma which have been demonstrated in this chapter to significantly inhibit VEGF-induced VEGFR-2 phosphorylation. Given that EGCG is also the predominant catechin found in green tea (El-Shahawi et al., 2012, Lee et al., 2014) it seems a sensible candidate polyphenol to further investigate the potential for polyphenol inhibition of pathological VEGF signalling *in vivo*.

Table 2.1: Summary of IC50 values achieved for inhibition of VEGF-induced VEGFR-2 phosphorylation by polyphenols

Polyphenol name	Structure	No -OH groups	IC50	Location of inhibition action
EGCG		8	96 nM	100 % VEGF ligand
Punicalagin		17	49 nM	90 % VEGF ligand 10 % cell
Ellagic acid		4	310 nM	90 % VEGF ligand 10 % cell
Urolithin D		4	200 nM	87 % VEGF ligand 13 % cell
Urolithin C		3	3.7 µM	100 % VEGF ligand
Urolithin A		2	54 µM	Not measured
Uroliothin B		1	251 µM	Not measured
Urolithin A-glc		5	No inhibition	Not measured
Urolithin B-glc		4	No inhibition	Not measured

2.5.2 Pomegranate polyphenol inhibition of VEGF signalling

The number of reports discussing the effects of pomegranate derived polyphenol on VEGF signalling inhibition is very limited. Where effects on VEGF signalling and its physiological responses have been investigated this has been primarily undertaken with pomegranate extract and, to a much more limited degree, with ellagic acid. VEGF protein secretion was reduced over 48 hrs following treatment with pomegranate extract but no measure of receptor expression or activation were performed (Sartippour et al., 2008). Pomegranate oil and fermented pomegranate both inhibited VEGF protein expression in a variety of cell types suggesting pomegranates have anti-VEGF potential, although phosphorylation status of VEGFR-2 was not measured (Toi et al., 2003). Also, as the treatment was with an extract and not individual compounds it is not known which component in the pomegranate extract caused the observed effect. In another experiment Bovine Aortic Endothelial Cells (BAECs) stimulated with VEGF for 1 minute after pre-treatment with ellagic acid at 5 and 10 μM for 24 hrs showed a significant reduction in VEGFR-2 phosphorylation. In the same assay phosphorylation of the downstream signalling molecule ERK was significantly reduced at 10 μM treatment (Labrecque et al., 2005). Considering VEGFR-2 phosphorylation peaks at 5-10 minutes post stimulation (Fearnley et al., 2016), 1 minute is a relatively short VEGF stimulation time, perhaps limiting the effect size achievable. A long pre-treatment time, 24 hours in this study, could lead to a change in the polyphenol profile in the media making conclusions about particular polyphenols difficult (Xiao and Hogger, 2015). For example in the caco-2 cell culture model, following 24 hours incubation with ellagic acid, the predominant compounds found in the media were dimethyl ellagic acid and dimethyl ellagic acid glucuronide, although free ellagic acid was still detected (Larrosa et al., 2006). Equally, it is difficult to compare the inhibitory capability as this study only models the polyphenol eliciting its effect via actions on the cell and does not come into contact with the VEGF ligand. The data presented in this chapter suggests that the pomegranate polyphenols punicalagin and ellagic acid have a rapid inhibitory effect on VEGFR-2 phosphorylation via action on the VEGF ligand itself. Following 5 minute stimulation with a pre-mixed treatment of

VEGF and the polyphenol, punicalagin was able to inhibit 50% of phosphorylation at 49 nM, more potent than any polyphenol reported to date. Ellagic acid was also a strong inhibitor of VEGF-induced VEGFR-2 phosphorylation albeit the effect was less potent than that of punicalagin with an IC50 value of 310 nM. This IC50 value for ellagic acid is similar to that previously reported by Cerezo et. al. of 230 nM (Cerezo et al., 2015).

The pomegranate polyphenol punicalagin has been found in the plasma of rats at concentrations up to 30 µg/ml. However, punicalagin has not been found in the plasma of any other species (Cerdá et al., 2003). Ellagic acid, which is found in the fruit itself and is a metabolite of punicalagin, is regularly reported to be found in plasma following pomegranate, pomegranate extract, and pure ellagic acid ingestion. Ellagic acid is also found in a wide variety of other foods such as walnuts and raspberries (Daniel et al., 1989). Predominantly the bioavailability studies in humans focus on interventions with ellagitannin containing foods or extracts. It has been suggested that both pomegranate juice and pomegranate extract induce a similar ellagic acid plasma concentration of 106 – 112 nM following intervention when the ellagitannin and ellagic acid profile is similar (ET 318 mg, EA 25 mg pomegranate juice; ET 330.4mg, EA 21.6mg pomegranate extract) (Seeram et al., 2004, Mertens-Talcott et al., 2006). In another study where 40 mg of ellagic acid only was fed to participants in the form of a capsule, over 6 times as much ellagic acid (662 nM) was detected in plasma (Hamad et al., 2009). This study suggests that feeding pure ellagic acid may elicit a larger EA C_{max} comparative to pomegranate or EA/ellagitannin combination intervention. However, more recently González-Sarrías et. al. demonstrated that EA plasma bioavailability is not changed when the EA dose is significantly increased with the low dose (279 mg ET, 25 mg EA) giving a EA plasma concentration of 74.8 nM while the high dose (130 mg punicalagin, 524 mg EA) resulted in an EA plasma concentration of 64.1 nM (González-Sarrías et al., 2015). Another study by Seeram et. al. also demonstrates ellagic acid is capable of reasonable plasma concentrations following a similar polyphenol dose containing 318 mg ellagitannins and 12 mg ellagic acid. In this study the ellagic acid peak plasma concentration was 60 nM (Seeram et al., 2006), which is very similar to the

concentrations achieved by González-Sarrías et. al. These studies demonstrate that ellagic acid has the potential to reach concentrations in plasma which were reported in this chapter to be significantly inhibitory of VEGF induced VEGFR-2 phosphorylation *in vitro*. This suggests, should the inhibitory effect be translatable to an *in vivo* setting, that EA could reach a concentration capable of inhibiting an effect.

Metabolism and bioavailability of ellagic acid metabolites must also be taken into account. Urolithin D and urolithin C with tetra- or tri-hydroxy groups respectively, are more hydrophilic and therefore less likely to pass through the epithelial barrier and into the plasma (Tammela et al., 2004), although urolithin C (Cerda et al., 2004) has been seen at reasonable concentration and urolithin C and D glucuronides were previously reported at high concentrations in plasma (79 and 87 µM respectively) (Pfundstein et al., 2014). However, urolithin A and B appear to be readily absorbed from the GI tract (Espín et al., 2007). The results presented in this chapter demonstrate that there is a negative impact of metabolism, be it hydrolysis or conjugation, on the ability of the polyphenol to inhibit VEGF-induced VEGFR-2 phosphorylation. Urolithin D was a potent inhibitor with an IC50 comparable to that of ellagic acid. However, urolithin D is thought to be readily metabolised to the consequent urolithins (Espín et al., 2007, Cerda et al., 2004). Urolithin C, which was shown to reach a up to a 1.8 µM concentration in plasma (Cerda et al., 2004), demonstrated significant inhibition *in vitro* of VEGF-induced VEGFR-2 at a similar concentration of 2 µM in this chapter. Urolithin A and B are only able to significantly inhibit VEGF induced VEGFR-2 phosphorylation at much higher concentrations than have been reported to date *in vivo.* However, urolithin quantification following intake of a high dose ellagic acid or punicalagin has not yet been conducted so it is unclear whether higher *in vivo* concentrations of the urolithins is achievable. The total urolithin concentration following dietary intervention has been shown to be as high as 18.6 µM and the inhibitory capacity of a combination of urolithins has not been investigated in this chapter, so the potential for a cumulative or collaborative inhibitory effect of urolithin treatment is unknown. However, in relation to whether the pomegranate polyphenols may be

able to inhibit VEGFR-2 phosphorylation *in vivo* it may be suggested that, while urolithin C has the potential to reach a similar dose to the 2 µM *in vitro* inhibitory dose, ellagic acid is the only pomegranate polyphenol which demonstrated potent inhibition of VEGF-induced VEGFR-2 at doses previously observed in plasma.

2.5.3 Mechanism of action for polyphenol inhibition of VEGF signalling

A further aim for this chapter was to identify a possible mechanism of action for the observed polyphenol inhibition of VEGF-induced VEGFR-2. The polyphenol could have elicited its inhibitory effects via 3 ways: 1) inhibition by interacting with the VEGF ligand; 2) interacting with the extracellular region of receptor or co-receptors or 3) inhibiting the VEGFR-2 tyrosine kinase activity intracellularly. Please see Figure 2.14 for a visual representation of these possible mechanisms of action.

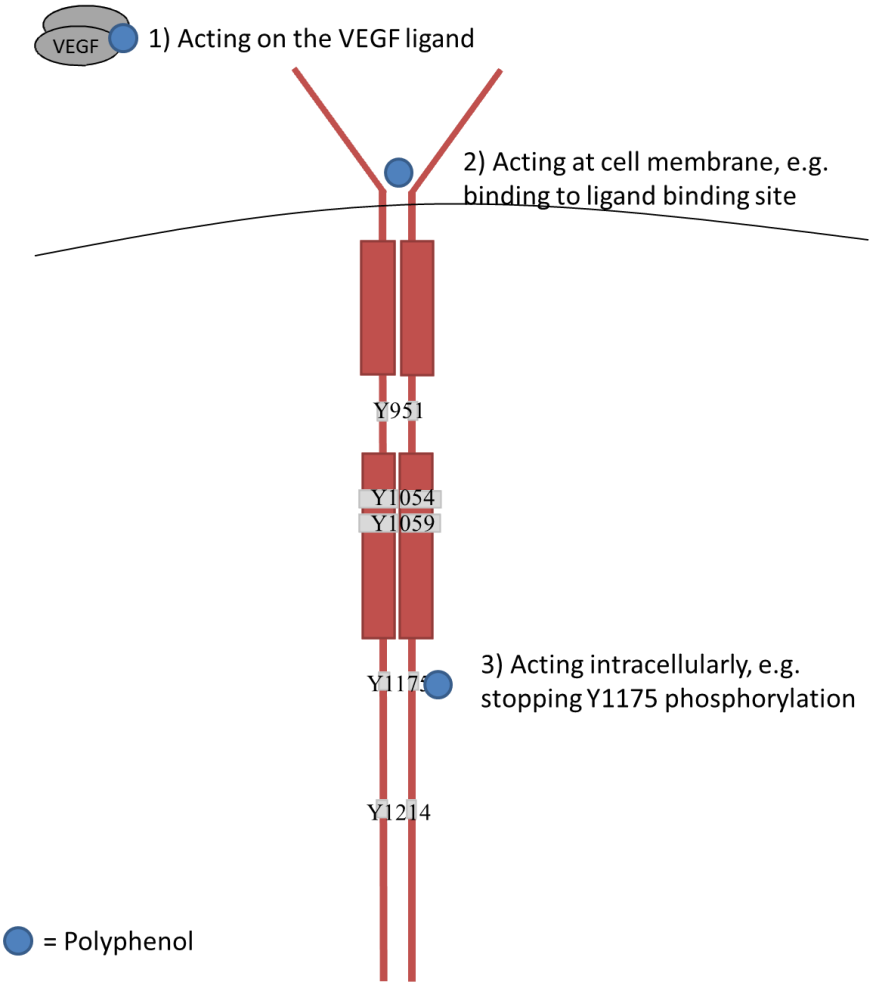


Figure 2.14: Diagram of the possible locations where the polyphenol are eliciting the VEGF inhibitory effects

Wang et. al. (Wang et al., 2012) observed inhibition of VEGFR-2 phosphorylation in HUVECs following 24 hr treatment with 5 and 10 µM treatment with ellagic acid and that the *in vitro* VEGFR-2 tyrosine kinase activity was strongly inhibited by ellagic acid with an IC50 of 25.8 nM. However, VEGFR-2 is a transmembrane receptor and the *in vitro* tyrosine kinase model assesses the effect on the VEGFR-2 protein in a free state. Considering the kinase domain of VEGFR-2 is intracellular this may explain the discrepancy between the strong inhibition elicited in the *in vitro* kinase assay which was not replicated in VEGFR-2 phosphorylation inhibition in a whole cell model. Through *in silico* modelling they hypothesised that this inhibition occurred due to interactions of ellagic acid with the ATP binding region of the VEGF-2 (intracellular location) but no demonstration that ellagic acid was entering the cell was provided limiting this conclusion. In contrast, Cerezo et. al (Cerezo et al., 2015) suggested that the polyphenols capable of inhibiting VEGFR-2 phosphorylation in the 5 minute model could exert their effect through interactions with the VEGF ligand. *In silico* modelling predicted that the inhibitory polyphenols were all able to bind to VEGF dimer close to the receptor binding site. Moreover, there was a strong correlation (r = 0.9445) between the predicted binding affinities and the IC50 value determined in the cell culture model. While the *in silico* model is a very simple binding prediction, with only a 1:1 VEGF (dimer):polyphenol input parameter it can be a useful tool for predicting a possible mode of action for further investigation. In 2002 Kondo et. al. demonstrated for the first time that a polyphenol was able to reduce the amount of tagged VEGF bound to HUVECs by around 40% following a 30 minute pre-treatment with EGCG (Kondo et al., 2002) and more recently it was reported that binding of tagged VEGF to HUVECs was reduced by 20% following 40 µM EGCG treatment (Moyle et al., 2015). Both of these papers suggest that EGCG has the potential to inhibit VEGFR-2 signalling through interactions with either the extracellular receptor domain to reduce VEGF/VEGFR-2 coupling or to the VEGF ligand. In this chapter it was demonstrated that all the polyphenols tested were required to interact with the VEGF ligand to be able to elicit the potent inhibition VEGF induced VEGFR-2 phosphorylation. Treatment of the cells with EGCG and urolithin C independently prior to VEGF stimulation did not result in any reduction of VEGFR-2 phosphorylation,

demonstrating that inhibition by these polyphenols can only occur when the polyphenol and VEGF are able to come into contact. A higher concentration was not tested as this was deemed supra-physiological. Treatment of the cells with punicalagin, ellagic acid and urolithin D independently prior to VEGF stimulation resulted in only 10 % inhibition of VEGFR-2 phosphorylation suggesting once again the potent inhibition of VEGF-induced VEGFR-2 phosphorylation only occurs when the polyphenol and VEGF can interact, although some inhibition through receptor/cell interactions can occur. Perhaps the *in silico* modelling by Wang et. al identifying the ATP binding region of the VEGFR-2 as a binding location of ellagic acid suggests a justification of this 10 % for ellagic acid. However, it demonstrates that the polyphenols are required to interact with the VEGF ligand to result in the potent and rapid inhibition of VEGF signalling discussed in this chapter.

2.6 Conclusion

The results presented in this chapter demonstrate that: 1) the dietary derived polyphenol EGCG, punicalagin and ellagic acid are able to potently inhibit VEGF-induced VEGFR-2 phosphorylation at nanomolar concentrations; 2) EGCG and ellagic acid were able to inhibit VEGF-induced VEGFR-2 phosphorylation at biologically relevant concentrations 3) metabolism of the pomegranate polyphenols reduced the inhibitory effect on VEGF-induced VEGFR-2 phosphorylation 4) all polyphenols demonstrating potent inhibition of VEGF-induced VEGFR-2 phosphorylation did so primarily through interactions with the VEGF ligand.

Considering that EGCG and ellagic acid have demonstrated potent and rapid inhibition of VEGF-induced VEGFR-2 phosphorylation at concentrations that can be found in plasma, both EGCG and ellagic acid are good candidates for further investigation into the potential health benefits from polyphenol inhibition of VEGF signalling. This will be explored in the next chapter, which will focus on the ability of EGCG and ellagic acid to inhibit VEGF-induced atherosclerosis in the ApoE-/- mouse model.

Chapter 3

Quantifying atherosclerosis in the VEGF injected ApoE-/- mouse following epigallocatechin gallate and ellagic acid intervention

3.1 Abstract

Intake of green tea and pomegranate products is associated with cardiovascular improvements. Angiogenesis is associated with atherosclerosis progression while intraperitoneal VEGF injection in animals increased atherosclerotic plaque size. The aim of this chapter is to investigate whether dietary intake of EGCG and EA can inhibit VEGF-induced and VEGF-independent atherosclerotic progression.

ApoE-/- mice received 3 weekly intraperitoneal injections of either 2 μg/kg body weight VEGF or mouse serum albumin (MSA) alongside feeding either a 1.8 g/kg pellet weight EGCG and EA supplemented or a control (non-supplemented) diet. Atherosclerotic plaque was quantified in the aortic sinus by histological analysis and cholesterol was measured using a biochemical analyser.

All mice developed significant increases in all parameters of plaque size over time in association with the atherogenic diet. Neither 3 weekly intraperitoneal VEGF injections nor diet type caused a significant change in plaque size in comparison to controls. Injection of VEGF + EGCG and EA resulted in reduced plaque thickness but did not alter overall plaque size in comparison to VEGF only injected animals. Total and LDL cholesterol plasma levels were significantly higher in the mice fed the EGCG and EA supplemented diet, regardless of injection type. VEGF injection was associated with reduced plasma triglycerides while polyphenol injection was associated with increased plasma triglycerides, although not at all time points.

In conclusion, 3 times 2 μg/kg body weight VEGF injections did not increase atherosclerosis plaque progression above MSA injected mice in the ApoE-/- model fed an atherogenic diet. Furthermore, EGCG and EA dietary supplementation at 1.8 g/kg pellet weight did not reduce atherosclerosis in the ApoE-/- model, irrelevant of injection type, and caused a small increase in plasma total cholesterol and LDL cholesterol levels however these levels were not significantly increased in comparison to baseline plasma lipid measurements. As the polyphenol dose of these compounds used was lower than in previous experiments where an anti-atherosclerotic effect was observed it suggests that lower doses may not be effective, at least in hypercholesterolaemic models.

3.2 Introduction

In chapter 2 it was demonstrated that the predominant polyphenols in both green tea and pomegranates were able to inhibit VEGF-induced VEGFR-2 phosphorylation. Two of these potent polyphenols, EGCG and EA, were able to significantly inhibit VEGF-induced VEGFR-2 phosphorylation at biologically relevant concentrations. Furthermore, these polyphenols elicited their inhibitory action primarily through interacting with the VEGF ligand and not the VEGF receptor or cell. In this chapter the current research into the role of VEGF in atherosclerotic progression will be discussed while the aim of this chapter is to identify whether the potent inhibition of VEGF-induced VEGFR-2 phosphorylation by EGCG and EA observed *in vitro* can be translated into *in vivo* inhibition of VEGF-induced atherosclerosis.

3.2.1 Atherosclerosis progression

Atherosclerosis causes symptomatic disease by reducing blood flow to the tissue either through direct narrowing of the artery (plaque growth) or plaque rupture causing a blood clot (thrombus) which can occlude at the location of the plaque or travel through the vascular network to another location. The atherosclerotic plaque can also weaken the artery which can increase the risk of aneurysm and vessel rupture. For a diagrammatic overview please see Figure 1.6.

Atherosclerosis starts with a thickening of the vessel wall through accumulation of smooth muscle cells and lipid laden macrophages, leading to fatty streaks in the aortic tissue. These further develop into large extracellular lipid pools causing significant growth of the lesion beyond the diffusion barrier leading to oxygen starvation and necrosis at the core. Fibrous cap formation and calcification also occur and new blood vessels are generated to supply the growing plaque. Increased vascularisation of the plaque leads to further instability of the vessel wall and an increased risk of plaque rupture and thrombus formation (Insull, 2009, Stary et al., 1995).

The ApoE-/- mouse model has a reduced ability to clear vLDL and LDL cholesterol from circulation and develops atherosclerosis, including the phases of disease

progression, in a similar manner to humans making the genotype an ideal model for investigating the disease (Nakashima et al., 1994, Pendse et al., 2009).

3.2.2 Role of angiogenesis and VEGF in atherosclerosis progression

As with cancer the growing atherosclerotic plaque requires the generation of new blood vessels, which are not required in healthy tissue, to continue the disease progression (Chen et al., 1999), stimulated through hypoxia associated signalling (Kuwahara et al., 2002). Histological analysis directly correlates the number of microvessels in the atherosclerotic plaque with the degree of severity of atherosclerosis, including the uptake of foam cells, tissue necrosis (Hiyama et al., 2010), intra-plaque haemorrhage, thrombosis, fibrous cap formation (Hiyama et al., 2010) and calcification (Demer and Tintut, 2008). Hypoxic signalling is known to upregulate the expression of VEGF, suggesting a critical role of VEGF in the atherosclerosis related angiogenesis (Hirota and Semenza, 2006). Furthermore, a correlation between circulating VEGF levels and critical lesion formation has been reported, while VEGF levels have been shown to be associated with increased risk of CVD (Kaess et al., 2016, Kimura et al., 2007), although not in all studies (Sandhofer et al., 2009, Alber et al., 2005). Increased VEGF, VEGFR-1 and VEGFR-2 expression, as well as higher microvessel density, has also been observed in atherosclerotic regions of arteries (Inoue et al., 1998). It was also shown that direct injection of VEGF into animal models induced atherosclerotic plaque growth by up to 10-fold (Celletti et al., 2001a, Celletti et al., 2001b). Vaccination against VEGFR-2 inhibited atherosclerotic progression (Hauer et al., 2007). However, another study in the same model reported inhibition of pan-VEGF receptor activity stimulated atherosclerosis progression. This may suggest that, while inhibition of angiogenesis associated VEGF signalling can have a limiting effect on atherosclerosis progression, that inhibition of all VEGF signalling may have the opposite effect. Furthermore, the negative side effects associated with anti-VEGF treatment which have emerged over the last few years (Tunon et al., 2009) may be mirrored in the ApoE-/- pan-VEGFR inhibitor study. These studies demonstrate that greater understanding about the role of VEGF in atherosclerosis is needed but do suggest that regulation of raised VEGF levels could prove to be an important treatment for atherosclerotic

disease. Furthermore, given the negative side effects with current anti-VEGF treatments, such as increased risk of thrombosis (Thulliez et al., 2014), there is room for an alternative therapy. Food bioactives could fulfil this role as they are able to be consumed in high amounts with fewer concerns about toxicity.

3.2.3 Polyphenol effects on VEGF and angiogenesis

More difficult to ascertain are the *in vivo* effects of polyphenols on VEGF and in turn VEGF associated angiogenesis. This is due to the low levels of naturally circulating VEGF and the many interconnected signalling pathways associated with VEGF induced angiogenesis *in vivo*. The majority of the research into the effects of polyphenols on VEGF signalling or indeed VEGF-induced angiogenesis has been performed using human cell lines, predominantly HUVECs or ex vivo tissue culture of aortic tissue from animals.

EGCG and EA effects on VEGF signalling

In HUVECs it has been observed that polyphenols can affect VEGF signalling through a number of different mechanisms, including alterations to gene and protein expression, VEGFR-2 expression or phosphorylation, and on downstream signalling events. EGCG has been reported to significantly inhibit VEGF mRNA expression (20 µM) (Kim et al., 2007a) and protein expression (100 µM) (Dann et al., 2009), VEGFR-2 protein expression (55 µM) (Shimizu et al., 2010) as well as the binding of labelled VEGF to HUVECs at 25 µM (Kondo et al., 2002). EA has been reported to inhibit phosphorylation of the downstream signalling partners of VEGFR-2, for example PLCγ, ERK and AKT, while EGCG is reported to inhibit phosphorylation of these molecules at comparatively low concentrations (2-10 µM) (Labrecque et al., 2005, Rodriguez et al., 2006). EGCG and ellagic acid have demonstrated potent and specific inhibition of VEGFR-2 phosphorylation following 5 minute treatment when the polyphenol and VEGF have been pre-mixed (Moyle et al., 2015, Cerezo et al., 2015) with the same observation being shown to be repeatable in chapter 2 of this thesis. The IC50 values for both EGCG and EA under this 5 minute model reported in chapter 2 of this thesis were 96 and 310 nM respectively. The strength of polyphenol inhibition also correlated to the predicted binding affinity of the

polyphenol for the VEGF ligand (Cerezo et al., 2015). Taking this into account with the observation from chapter 2 that the potent inhibition of VEGFR-2 phosphorylation was not possible if the polyphenol and VEGF did not come into contact, it suggests EGCG and ellagic acid inhibit VEGF-induced VEGFR-2 phosphorylation by interacting with the VEGF ligand.

EGCG and EA effects on angiogenesis

To accurately determine whether new vessel formation can be affected there are two key *in vitro* models: 1) HUVECs are seeded into an extracellular matrix and stimulated with VEGF [49] +/- polyphenol (DeCicco-Skinner et al., 2014); 2) *ex vivo* aortic tissue is embedded into an extracellular matrix and stimulated to grow with VEGF (Baker et al., 2012) +/- polyphenol. Both ellagic acid and EGCG have been reported to inhibit tube formation by HUVECs. Ellagic acid has demonstrated anti-angiogenic effects, with 5 µM treatment inhibiting 38 % (Labrecque et al., 2005) and 10 µM inhibiting 85 % (Wang et al., 2012) of tube formation in a VEGF unstimulated model. VEGF stimulated tube formation was inhibited by EGCG treatment and inhibited both the number of branch points and the tube length in this assay at 20 µM (Liu et al., 2008). The aortic ring assay measures the number of new microvessels which sprout from the *ex vivo* aortic tissues in culture under the experimental conditions and is a model of arterial angiogenesis. Ellagic acid at 5 µM was able to potently inhibit angiogenesis in *ex vivo* aortic rings after 48-72 hours (Wang et al., 2012). EGCG has yet to be tested, however a high dose of catechin did not significantly alter the new vessel growth (Negrão et al., 2013). There are also *in vivo* angiogenesis assays such as the chick chorioallantoic membrane (CAM) or matrigel plug assay which use the natural blood vessel system of the animal to determine if angiogenesis can be stimulated or inhibited by the treatment. An extract of green tea catechins, of which EGCG is the predominant polyphenol, at 1 % (Maiti et al., 2003) and pomegranate extract at doses as low as 0.1 µg (Toi et al., 2003), as well as pure ellagic acid treatment at 2.5 µM (Wang et al., 2012) were able to significantly inhibited angiogenesis in the CAM assay. A number of polyphenols have demonstrated anti-angiogenic potential in the matrigel plug model including significant effects by 100 µM catechin (Negrão et al., 2013) and

EGCG (Li et al., 2013) measured by the amount of haemoglobin content of the plug however ellagic acid has yet to be investigated in this manner.

3.2.4 The effect of green tea and pomegranate polyphenols on CVD

Epidemiology and human intervention trials

The association between reduced CVD risk of and increased polyphenol consumption has been reported for many years (Hertog et al., 1993, Knekt et al., 1996, Arts and Hollman, 2005, Lai et al., 2015, Keli et al., 1996, Arts et al., 2001b). A recent meta-analysis focussed on the cardiovascular related effects of green tea found that higher consumers of green tea had a reduced risk of myocardial infarction, stroke, and, in the case of those drinking ≥10 cups a day, a reduced LDL level (Pang et al., 2016). Other studies found a significant association between green tea consumption and blood pressure reduction, particularly in participants with an elevated baseline systolic BP (Khalesi et al., 2014). A review into 133 polyphenol intervention studies found that cocoa or chocolate and green tea were associated with positive effects on CVD risk factors by reducing LDL cholesterol levels in participants (Hooper et al., 2012). A number of studies describe improvements to blood pressure (Mathew et al., 2012), carotid intima media thickness (Aviram et al., 2004) and reduced stress induced ischemia (Sumner et al., 2005) in response to pomegranate intervention while a meta-analysis of 12 studies found no significant effect of on lipid levels (Sahebkar et al., 2016). However, a critical evaluation of the intervention trials into pomegranate effects on cardiovascular disease highlighted the need for more rigorous and controlled interventions with a clear dosing strategy. This clearly suggests caution should be used when interpreting the individual study data (Vlachojannis et al., 2015).

Animal intervention trials

Animal studies can be used to assess the effects of diets supplemented with particular compounds on atherosclerotic plaque morphology and degree of atherosclerosis. (Rouanet et al., 2010, Yamakoshi et al., 1999, Loke et al., 2010, Kleemann et al., 2011, Shen et al., 2013, Chanet et al., 2012) Mice which drank green tea were found to have significantly reduced lipid deposition in the aortic

wall (Rouanet et al., 2010) and around 30 % less atherosclerotic plaque compared to control groups, with an associated reduction in VEGF expression in the plaque itself (Kavantzas et al., 2006). Hyperlipidaemic mice (Miura et al., 2001, Minatti et al., 2012) and rabbits (Tijburg et al., 1997) orally supplemented with a green tea extract had up to a 35 % reduction in atherosclerosis compared to control groups. Equally, intraperitoneal injection of EGCG has been demonstrated to inhibit progressive atherosclerosis (Ramesh et al., 2010) although it did not reduce established plaque (Chyu et al., 2004). A reduction in lipid deposition in the aortic arch caused by EGCG supplementation (35.5 %) was enhanced through nano-encapsulation of the polyphenol (74.1 %), which might be explained by overcoming the low stability of EGCG *in vivo* (Hong et al., 2014). Supplementation of pomegranate juice (Aviram et al., 2008) and pomegranate fruit extracts to the diet has demonstrated significant atherosclerotic plaque reduction by around 40 % in hypercholesteraemic mice (de Nigris et al., 2007). The pomegranate flowers and pomegranate peel contained the most anti-atherogenic compounds (Aviram et al., 2008). Furthermore, a 2 % ellagic acid supplementation to the diet of diabetic rats reduced intimal thickness, lipid deposition, and collagen inclusion in the aortic arch (Rani et al., 2013) while 1 % ellagic acid supplementation to the diet of rabbits completely negated the atherogenic effects of the cholesterol supplemented diet (Yu et al., 2004).

3.2.5 Objectives

The aim for this chapter is to determine whether EGCG and EA, two potent inhibitors of VEGF-induced VEGFR-2 phosphorylation *in vitro*, are able to inhibit VEGF-induced atherosclerosis *in vivo*. In previous studies intraperitoneal injection of VEGF induced atherosclerosis in both mice and rabbits while dietary intervention with EGCG and EA rich foods, or the compounds themselves, inhibited atherosclerotic progression. This chapter will present a study investigating whether an EGCG and EA supplemented diet can affect atherosclerosis in the ApoE-/- mouse which has undergone 3 times VEGF intraperitoneal injection. In a further experimental arm the VEGF will be pre-mixed with the polyphenol to determine if the *in vitro* observations can be directly translated to an *in vivo* environment.

This leads to a number of hypothesis: 1) VEGF injection will induce significant atherosclerosis above a mouse serum albumin (MSA) injected control; 2) dietary supplementation with EGCG and ellagic acid, potent *in vitro* inhibitors of VEGF-induced VEGFR-2 phosphorylation, will inhibit VEGF-induced atherosclerosis; 3) dietary supplementation with EGCG and EA will be able to limit endogenous VEGF signalling associated with atherosclerotic progression; 4) mixing EGCG and EA with VEGF prior to intraperitoneal injection will completely ablate any VEGF stimulation of atherosclerosis.

3.3 Material and methods

3.3.1 Animal diet production

Pellets were manufactured by TestDiet (Test Diet Limited, London, UK). EGCG was ordered from Toronto Research Chemicals, Canada. EA was ordered from Acros Chemicals, Belgium. All additives and complete diets were subject to nutritional review by TestDiet specialists. The experimental diet was the TestDiet 57BB Paigen diet (15.5 % fat, 1.25 % cholesterol, 0.5 % sodium cholate). For reference, from here this diet will be called the 'basic diet'. EGCG and EA were sent to the manufacturer for mixing with the 57BB recipe and this new diet was named by the manufacturer as 5W70. Both compounds were added at 1.8 g/kg total pellet weight providing a 0.18% pellet content of each polyphenol. For reference, from here this diet will be called the 'EGCG/EA diet'. The AIN-93G diet was also produced to be used during the acclimatisation period. All pellets underwent a slow drying process to reduce the risk of compound breakdown and were sealed in airtight bags then irradiated for sterility.

3.3.2 Animal condition and experimental design

The experiments described here were conducted in compliance with the European Union regulations concerning the protection of experimental animals and with the UK Home Office Animals (Scientific Procedures) Act of 1986 under the personal licence I181618BE and project licence 80/2533.

100 mice were delivered in 5 batches aged 3 weeks and were kept in acclimatisation on AIN-93G. At age 4 weeks they were switched to the basic diet but remained in acclimatisation for a further week. All mice continued on the basic diet until aged 8 weeks. 10 mice were culled at 8 weeks for a baseline measurement. At 8 weeks of age all non-baseline mice underwent 3 x weekly injections of a 2 µg/kg body weight of either VEGF, MSA or VEGF combined with EGCG and EA. At the time of the first injection mice either remained on the basic diet or were switched to the EGCG/EA diet until they underwent terminal anaesthesia. 9 mice from each group were culled at age 11 weeks and a further 9 from each group at 14 weeks of age (it should be noted 3 mice died prematurely

before any intervention and these were not included in the final data). All Mice were culled by terminal anaesthesia and cardiac puncture/perfusion. All animals were kept in individually ventilated cages in a 22 °C controlled temperature room with 55 % humidity and a 12 h light-dark cycle. Bedding was changed every two weeks. Cages were checked daily by staff and individual mice were health checked and weighed 3 times a week. Following intraperitoneal injection mice were checked for signs of inflammation around the injection site. Mice were provided food and water ad libitum using the cage hopper and water bottle. See Table 3.1 for study overview.

Table 3.1: Outline of diet type, injection type, and number of animals per time point in each treatment if the animal intervention trial

Treatment name	Diet type (4-8 weeks)	Injection type	Diet type (8-14 weeks)	No. culled at 8 weeks	No. culled at 11 weeks	No. culled at 14 weeks
Baseline	57BB (Paigen diet)	n/a	n/a	10	n/a	n/a
Basic diet + MSA injection	57BB (Paigen diet)	MSA	57BB (Paigen diet)	n/a	8	9
Basic diet + VEGF injection	57BB (Paigen diet)	VEGF	57BB (Paigen diet)	n/a	9	9
EGCG/EA diet +MSA injection	57BB (Paigen diet)	MSA	5W70	n/a	9	9
EGCG/EA diet + VEGF injection	57BB (Paigen diet)	VEGF	5W70	n/a	9	8
Basic diet + VEGF/EGCG/EA injection	57BB (Paigen diet)	VEGF/ EGCG/ EA	57BB (Paigen diet)	n/a	9	8

3.3.3 Monitoring of body weight and pellet consumption

Body weights were measured 3 times a week to monitor animal health and growth. The body weights of all animals based on injection type or diet type were combined to determine whether there were significant differences in the growth rates between treatment types. The body weights from the mice that did not complete the intervention were excluded.

To determine the daily intake of each diet the pellet consumption was monitored. Upon receiving a new diet the cages were changed and the initial pellet weight recorded. The pellets in the cage were transferred to a sterilised pot for weighing three times a week. Where required the mice were provided with further pellets of a recorded weight. It was noticed in week 9 that the mice were shredding their diet and creating food stores under the cage bedding, leading to an inaccurate assumption of diet intake. From this point forward a once weekly measurement of solid and powder diet was undertaken. Briefly, all mice were transferred to a clean cage after which the entire bedding, including the shredded diet, was sieved through an autoclaved kitchen sieve. This allowed the diet to fall through while the bedding remained in the sieve. The diet was weighed and the bedding, diet and mice were returned to the home cage.

3.3.4 Intraperitoneal injection of VEGF, mouse serum albumin or VEGF/PP combination

At age 8, 9, and 10 weeks each mouse underwent an intraperitoneal injection of 200 µl volume. Briefly, mice were transferred to a clean cage for procedure. Prior to injection the VEGF stocks were made to 200 µg/ml concentration while mouse serum albumin (MSA) was made to a 40 µg/ml stock concentrations and were 0.22 µm filter sterilised and stored at -80 until required. Immediately prior to injection a final stock solutions of 0.4 µg/ml VEGF and MSA were made up in sterile PBS. Mice were weighed on the morning of the injections to give an accurate body weight, and an excess of individual injection solution of VEGF and MSA were made to a concentration allowing for delivery of 2 µg/kg body weight in a 200 µl volume. Mice were injected with 200 µl solution by maintaining a tight scruff, gently tilting the animal so the head is lower than the flank, and injecting the full volume into the intraperitoneal sack. Upon removal the syringe tip was twisted to seal the wound, the mouse was returned to the home cage. The excess injection sample was snap frozen on dry ice to be used to determine the VEGF stimulatory capacity of the injection sample. The order of mice and cages to be injected was rotated over the three repeat injections to remove any impact of injection stability.

3.3.5 Quantification of VEGF activity of injection treatments

HUVECs were grown to confluency to be ready for the day of injection. Samples snap frozen at the time of injection were added to EBM-2 media to give a final VEGF concentration of 25 ng/ml as was standard in the cell culture experiments in chapter 2. A fresh VEGF positive control was also made immediately prior to the HUVEC treatment to compare the activity of the VEGF injection samples to. The activity of the MSA injection samples at 25 ng/ml was also tested. Each treatment was added to a well of confluent HUVECs for 5 minutes at 37°C, 5% CO_2 after which the cells were lysed and the proteins extracted. Total protein content was determined by using the BCA assay (Chapter 2.3.5), the samples were normalised to the same total protein content and the VEGFR-2 phosphorylation quantified in comparison to the fresh VEGF treatment by ELISA following the manufacturer's instructions as specified in chapter 2.3.6 (7335, Cell Signalling Technology Inc., New England Biolabs)

3.3.6 Terminal anaesthesia and tissue collection

Mice underwent terminal anaesthesia, cardiac puncture and saline perfusion at age 8, 11 and 14 weeks. Mice were anaesthetised using isoflurane and anaesthesia was maintained until confirmation of death. Complete anaesthesia was confirmed by a lack of muscle reflex following a physical pinch, after which the fur, peritoneum and diaphragm were cut to allow access to the heart. A 1 ml syringe with a 25 gauge, 16mm syringe tip was flushed with EDTA saline and was inserted directly into the apex of the heart. Once in place the blood was gently removed from the heart in time with the heart contractions to remove the total blood. Following blood collection the right ventricle was cut and a new syringe containing saline + EDTA was inserted into the apex of the heart. The animal was perfused with saline + EDTA until the saline came out clear and organ colour indicated no blood remained. Death was confirmed by beheading. The blood was spun at 3,500 x g for 10 minutes to separate erythrocytes and plasma, and the plasma transferred to a new tube. The plasma and the erythrocytes/buffy coat were snap frozen in dry ice. The left lateral lobe of the liver, right kidney and the aorta were immediately placed into RNALater and stored on wet ice. The heart, including the aortic arch, and the eyes

were placed into 4 % paraformaldehyde and stored on wet ice. The brain, spleen, intestine and stomach (including faeces), left kidney, lungs, visceral fat and remaining lobes of liver were snap frozen in dry ice. For long term storage the sample on wet ice were transferred to a -20 °C freezer and the samples on dry ice were stored at -80 °C.

3.3.7 Quantification of atherosclerotic lesions in aortic arch

Atherosclerotic lesions size was quantified by staining for lipid deposition across the aortic sinus. Following fixation the hearts were stored to a 30% sucrose solution for up to 1 week at 4 °C. The hearts were cleaned of any other tissue and a cut was made with a scalpel to remove the lower half of the heart. After this the heart was placed into a 1:1 solution 30% sucrose:optimal cutting temperature (OCT) solution for 30 minutes or until the hearts had sunk to bottom of the vessel. The hearts were then placed into OCT filled square moulds and snap frozen on dry ice. Serial 5 µm cryosections across the aortic sinus were performed on a cryostat (Cryo-Star NX70, Thermo Scientific) cooled to -20 °C. Sections began when the start of all three valves could be seen or a leaflet of one of the valves become visible, as demonstrated in Figure 3.1, and ended when all three valves had disappeared. Each section was collected across this region on 12 slides which lead to each slide containing serial sections at 60 µm distance from one another. Slide 3 was taken and stained with oil red O and haemotoxylin and the morphological features observed through this staining can be seen in Figure 3.2. Images of the plaque were taken with a colour camera attached to a BX60 Olympus microscope. Quantification of atherosclerotic plaque was performed in 3 ways. 1) Atherosclerotic plaque area as both the percentage of the total lumen area and area in μm^2 of the atherosclerotic plaque; 2) Atherosclerotic plaque circumferential expansion as both the percentage and distance in µm the plaque expanded along the lumen circumference; and 3) the maximal plaque thickness, presented as the distance in µm from the lumen wall to the end of the plaque at the thickest portion of plaque. All quantification was performed blinded using the imageJ software and a diagrammatic representation of how these measurements were undertaken for each section can be seen in Figure 3.3.

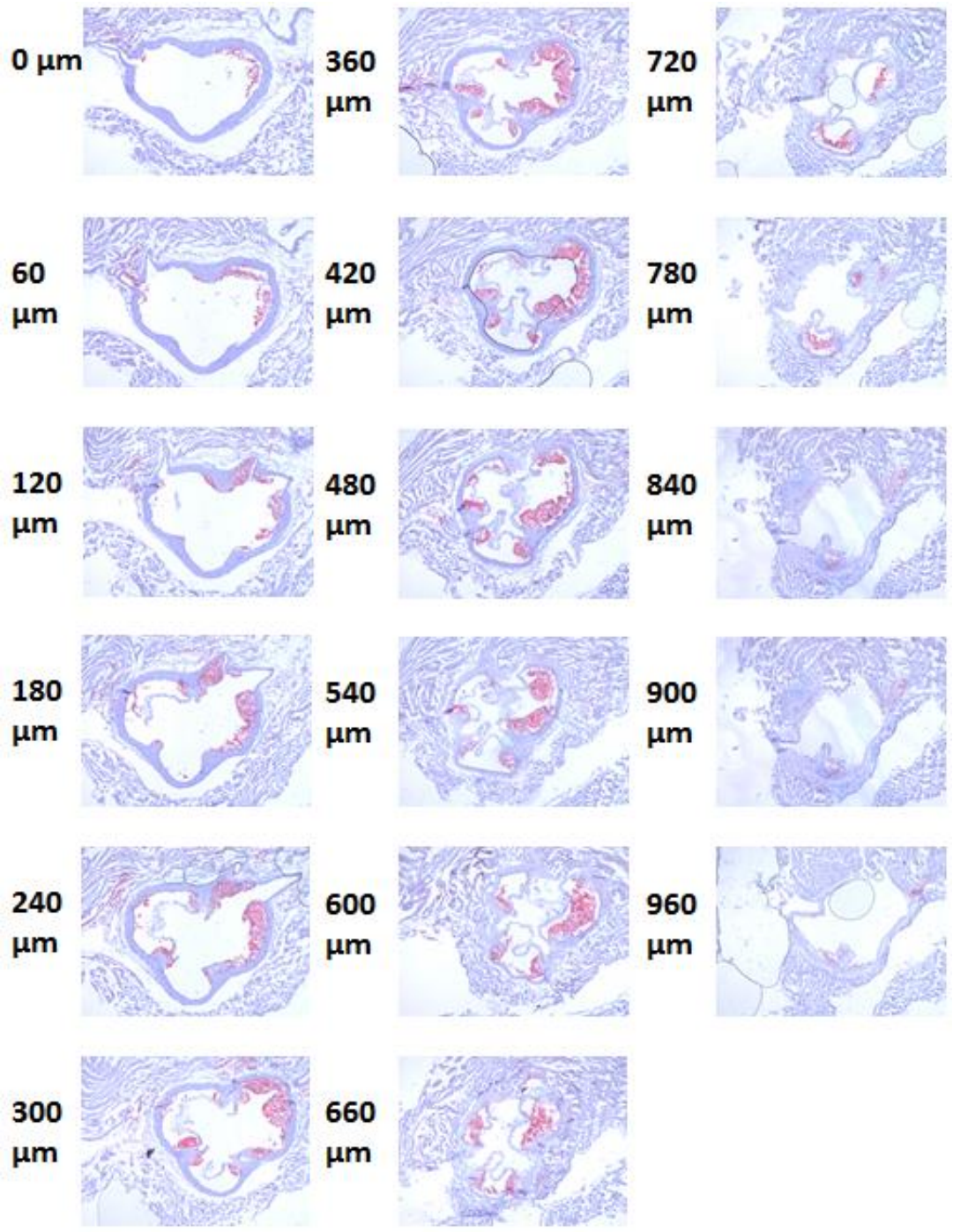


Figure 3.1: Representative cryosections stained with Oil Red O and haemotoxylin across the aortic sinus from one mouse.

The actual size shown in each image is 1.8 mm (width) x 1.3 mm (length).

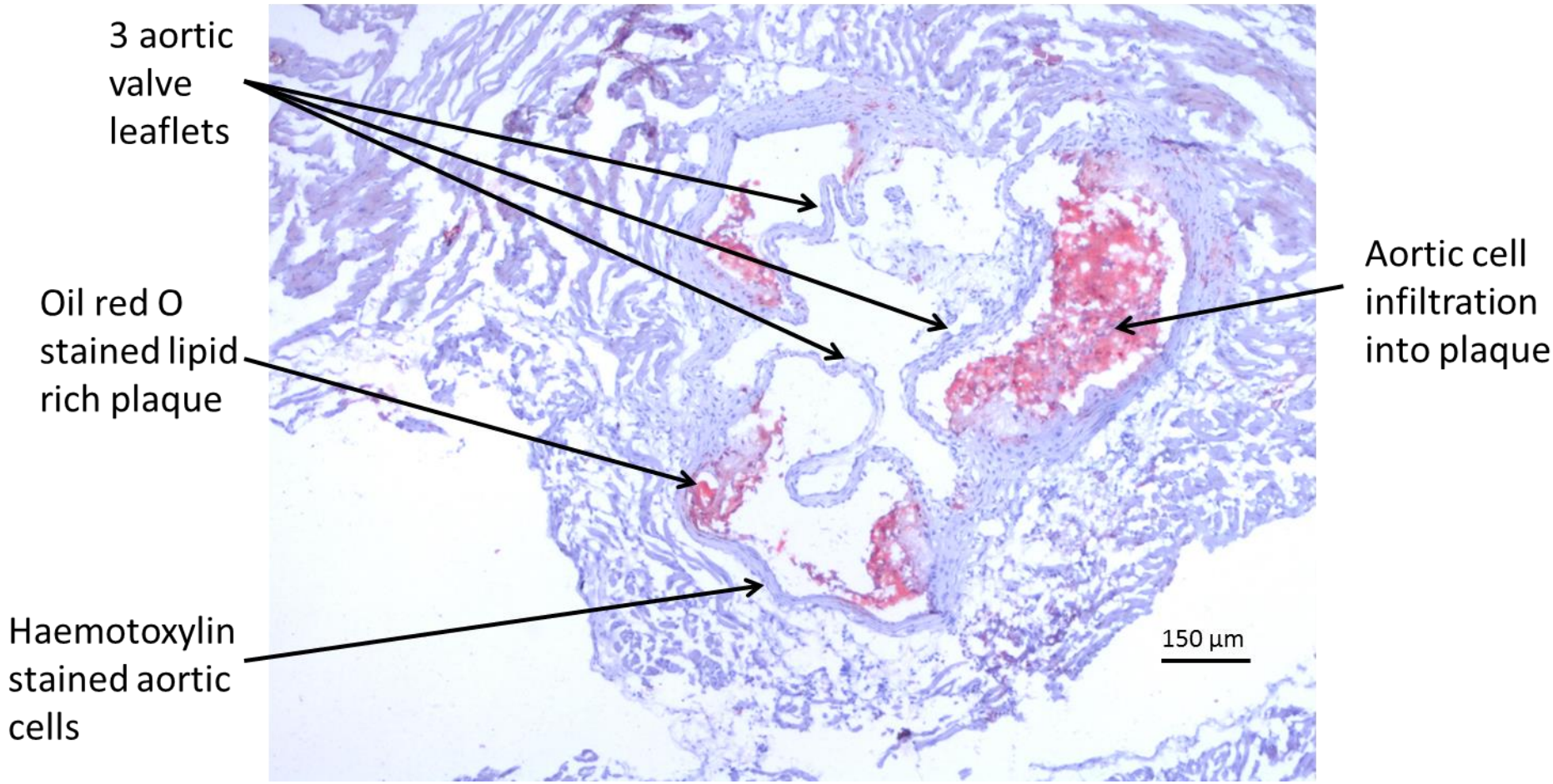


Figure 3.2: Example of the aortic sinus with labelled morphological features

The actual size shown in the image is 1.8 mm (width) x 1.3 mm (length). .

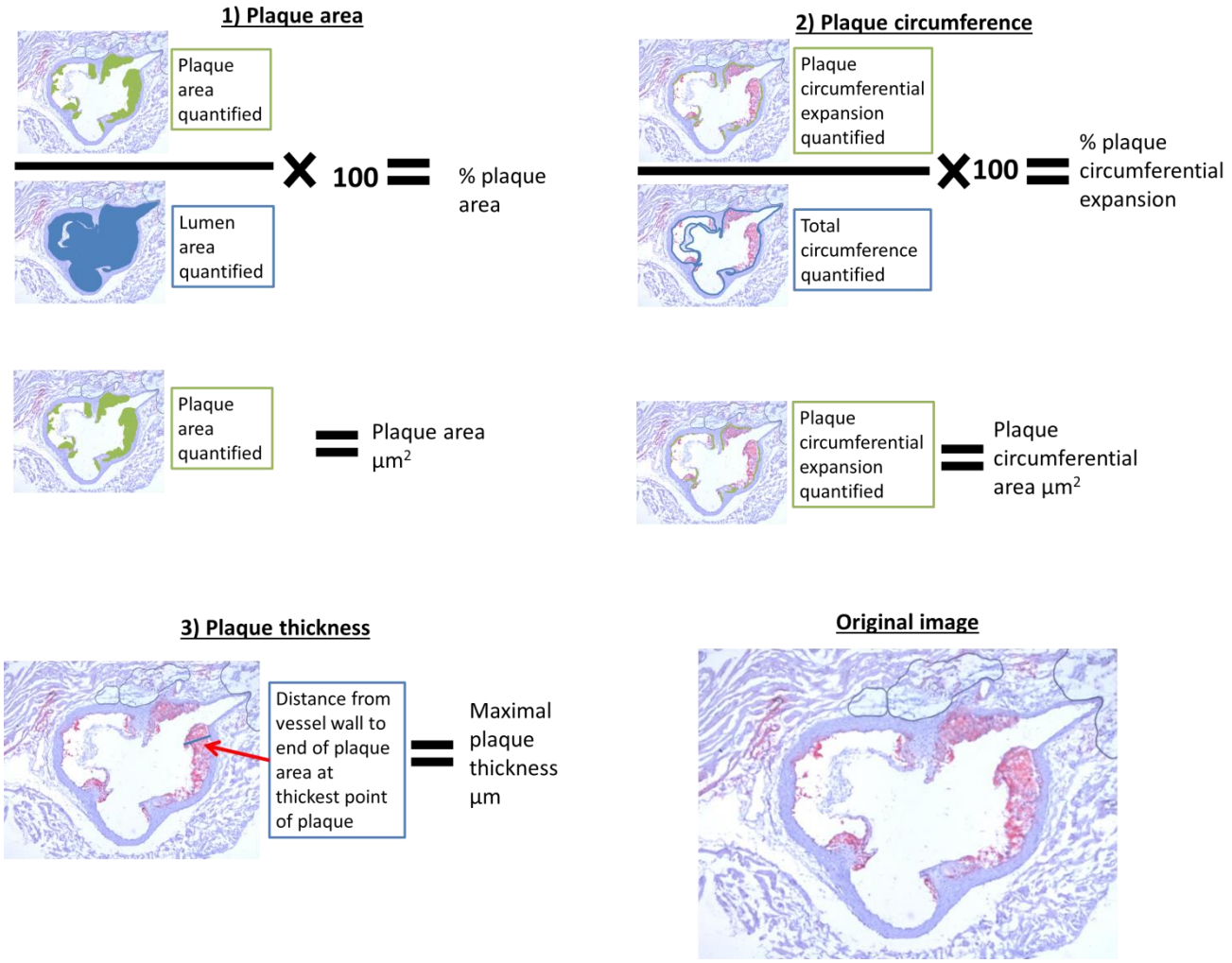


Figure 3.3: Diagrammatic representation of plaque size measurements.

3.3.8 Quantification of plasma lipid levels

Following storage of the plasma at -80 °C the samples were defrosted and total cholesterol, LDL cholesterol, and triglycerides were quantified using the Randox RX Daytona^{+} biochemical analyser (Randox Inc., Ireland) using the recommended Randox kits for each assay. Prior to initiation of analysis the machine was cleaned, primed and calibrated. Briefly, a minimum of 200 µl of plasma was transferred to a bioanalyser cup and placed into the sample chamber. The appropriate reagents and cleaning solutions were placed into the reagent chamber. For total cholesterol and triglyceride analysis only 1 reagent was needed (R1) and quantification was performed by colorimetric assay. For LDL cholesterol analysis 2 reagents were required (R1 and R2) and quantification was performed through a direct clearance method of analysis.

3.3.9 Quantification of polyphenols in plasma

Polyphenols were extracted from plasma following the protocol as previously described (García-Villalba et al., 2016). Briefly 600 µl acetonitrile:formic acid (98:2 v:v) was added to 200 µl plasma. 6,7, di-hydroxycoumarin was added to each sample to create a 0.1 ppm final concentration once reconstituted. Each sample was vortexed for 2 minutes, placed in a sonication bath at room temperature for 10 minutes. Samples were then centrifuged at 14,000 x g at 4 °C for 10 minutes and the supernatant transferred to a 2 ml tube. Samples were evaporated at 45 °C in a centrifugal evaporator until only a small amount of reagent remained. Following this, samples were dried to complete dryness at room temperature in a centrifugal evaporator. High (200 nM) and low (1 µM) QC samples were also extracted alongside the samples. Sample tube were made airtight and kept at -20 °C until shipping. Samples were shipped to Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas (CEBAS-CSIC) in Murcia for analysis. All the dried samples were re-suspended in 100 µL of MeOH and filtered through a 0.22 µm PVDF filter before analysis. 0.1 ppm of chrysin was added as internal standard immediately prior to LC-MS analysis. Samples were analysed using an Agilent 1290 Infinity UPLC system coupled to the 6550 Accurate-Mass quadrupole TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany)

using an electrospray interface with Jet Stream technology. Briefly, separation was performed using a reverse phase Poroshell 120 EC-C18 column (3 × 100 mm, 2.7 µm; Agilent) operating at 30 °C. Solvent A was water:formic acid (99.9:0.1 v/v) and solvent B was acetonitrile:formic acid (99.9:0.1 v/v) using the gradient program as follows: 0–3 min, 5–15 % B; 3–11 min, 15–30 % B; 11–15 min, 30–50 % B, 15–21 min, 50–90 % B, followed by 21- 26 min 5 % B to re-equilibrate the column. The flow rate was 0.4 mL/min and the injection volume was 3 µL. The electrospray interface conditions were as follows: gas temperature 280 °C, drying gas 9 L/min, nebulizer 45 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. Spectra were acquired in single MS mode with m/z range of 100–1100, negative polarity, and an acquisition rate of 1.5 spectra/s. Internal mass calibration by simultaneous acquisition of reference ions and mass drift compensation was used for obtaining low mass errors. Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00 Agilent Technologies). A list of all potential compounds to be found following EGCG or EA consumption which were searched for in the analysis can be seen in Table 3.2 and Table 3.3 respectively.

Table 3.2: List of potential compounds searched for in mice plasma samples to be found in plasma following epigallocatechin gallate consumption, their molecular formula and the molecular mass (M_r).

Compound	Molecular formula	Exact mass
EGC	$C_{15}H_{14}O_7$	305.0667
EGC glcA	$C_{21}H_{22}O_{13}$	481.0988
EGC sulfate	$C_{15}H_{14}O_{10}S$	385.0235
Methyl EGC	$C_{16}H_{16}O_7$	319.0823
Methyl EGC glcA	$C_{22}H_{24}O_{13}$	495.1144
Methyl EGC sulfate	$C_{16}H_{16}O_{10}S$	399.0391
EGCG	$C_{22}H_{18}O_{11}$	457.0776
EGCG glcA	$C_{28}H_{26}O_{17}$	633.1097
EGCG sulfate	$C_{22}H_{18}O_{14}S$	537.0344
Methyl EGCG	$C_{23}H_{20}O_{11}$	471.0933

Compound	Molecular formula	Exact mass
Methyl EGCG glcA	$C_{29}H_{28}O_{17}$	647.1254
Methyl EGCG sulfate	$C_{23}H_{20}O_{14}S$	551.0501
1-trihydroxy-phenyl-3-trihydroxyphenylpropan-2-ol	$C_{15}H_{16}O_7$	307.0823
1-trihydroxy-phenyl-3-trihydroxyphenylpropan-2-ol glcA	$C_{21}H_{24}O_{13}$	483.1144
1-trihydroxy-phenyl-3-trihydroxyphenylpropan-2-ol sulfate	$C_{15}H_{16}O_{10}S$	339.0391
1-dihydroxyphenyl-3-tri-hydroxyphenylpropan-2-ol	$C_{15}H_{16}O_6$	291.0874
1-dihydroxyphenyl-3-tri-hydroxyphenylpropan-2-ol glcA	$C_{21}H_{24}O_{12}$	467.1195
1-dihydroxyphenyl-3-tri-hydroxyphenylpropan-2-ol sulfate	$C_{15}H_{16}O_9S$	323.0442
5-dihydroxyphenyl-4-hydroxyvaleric acid	$C_{11}H_{14}O_5$	225.0768
5-dihydroxyphenyl-4-hydroxyvaleric acid glcA	$C_{17}H_{22}O_{11}$	401.1089
5-dihydroxyphenyl-4-hydroxyvaleric acid sulfate	$C_{11}H_{14}O_8S$	305.0337
5-dihydroxyphenyl-γ-valerolactone	$C_{11}H_{12}O_4$	207.0663
5-dihydroxyphenyl-γ-valerolactone glcA	$C_{17}H_{20}O_{10}$	383.0984
5-dihydroxyphenyl-γ-valerolactone sulfate	$C_{11}H_{12}O_7S$	287.0231
3-dihydroxyphenyl propionic acid	$C_9H_{10}O_4$	181.0506
3-dihydroxyphenyl propionic acid glcA	$C_{15}H_{18}O_{10}$	357.0827
3-dihydroxyphenyl propionic acid sulfate	$C_9H_{10}O_7S$	261.0074
4-dehydroxy EGC	$C_{15}H_{14}O_6$	289.0718
4-dehydroxy EGC glcA	$C_{15}H_{14}O_9S$	369.0286
4-dehydroxy EGC sulfate	$C_{21}H_{22}O_{12}$	465.1038
5-trihydroxyphenyl-4-hydroxyvaleric acid	$C_{11}H_{14}O_6$	241.0718
5-trihydroxyphenyl-4-hydroxyvaleric acid glcA	$C_{17}H_{22}O_{12}$	417.1038
5-trihydroxyphenyl-4-hydroxyvaleric acid sulfate	$C_{11}H_{14}O_9S$	321.0286
5-trihydroxyphenyl-γ-valerolactone	$C_{11}H_{12}O_5$	223.0612
5-trihydroxyphenyl-γ-valerolactone glcA	$C_{17}H_{20}O_{11}$	399.0933

Compound	Molecular formula	Exact mass
5-trihydroxyphenyl-γ-valerolactone sulfate	$C_{11}H_{12}O_8S$	303.018
5-dihydroxyphenyl valeric acid	$C_{11}H_{14}O_4$	209.0819
5-dihydroxyphenyl valeric acid glcA	$C_{17}H_{22}O_{10}$	385.114
5-dihydroxyphenyl valeric acid sulfate	$C_{11}H_{14}O_7S$	289.0387
5-hydroxyphenyl valeric acid	$C_{11}H_{14}O_3$	193.087
5-hydroxyphenyl valeric acid glcA	$C_{17}H_{22}O_9$	369.1191
5-hydroxyphenyl valeric acid sulfate	$C_{11}H_{14}O_6S$	273.0438
5-trihydroxyphenyl valeric acid	$C_{11}H_{14}O_5$	225.0768
5-trihydroxyphenyl valeric acid glcA	$C_{17}H_{22}O_{11}$	401.1089
5-trihydroxyphenyl valeric acid sulfate	$C_{11}H_{14}O_8S$	305.0337

EGC = epigallocatechin; EGCG = epigallocatechin gallate; GlcA = glucuronide

Table 3.3: List of potential compounds searched for in mice plasma samples to be found in plasma following ellagic acid consumption, their molecular formula and the molecular mass (M_r).

Compound	Molecular formula	Exact mass
UroA glcA	$C_{19}H_{16}O_{10}$	403.0671
UroA sulfate	$C_{13}H_8O_7S$	306.9918
UroA sulfoglcA	$C_{19}H_{16}O_{13}S$	483.0239
UroA	$C_{13}H_8O_4$	227.0350
Methyl UroA	$C_{14}H_{10}O_4$	241.0506
Methyl UroA glcA	$C_{20}H_{18}O_{10}$	417.0827
Methyl UroA sulfate	$C_{14}H_{10}O_7S$	321.0074
UroB glcA	$C_{19}H_{16}O_9$	387.0722
UroB sulfate	$C_{13}H_8O_6S$	290.9969
UroB-sulfoglcA	$C_{19}H_{16}O_{12}S$	467.029
UroB	$C_{13}H_8O_3$	211.0401
UroC glcA	$C_{19}H_{16}O_{11}$	419.062
UroC sulfate	$C_{13}H_8O_8S$	322.9867
UroC sulfoglcA	$C_{19}H_{16}O_{14}S$	499.0188
UroC	$C_{13}H_8O_5$	243.0299

Compound	Molecular formula	Exact mass
Methyl UroC glcA	$C_{20}H_{18}O_{11}$	433.0776
Methyl UroC sulfate	$C_{13}H_8O_8S$	322.9867
Methyl UroC	$C_{14}H_{10}O_5$	257.0455
Dimethyl UroC glcA	$C_{21}H_{20}O_{11}$	447.0933
Dimethyl UroC	$C_{15}H_{12}O_5$	271.0612
UroM6 glcA	$C_{19}H_{16}O_{12}$	435.0569
UroM6 sulfate	$C_{13}H_8O_9S$	338.9816
UroM6	$C_{13}H_8O_6$	259.0248
Methyl UroM6 glcA	$C_{20}H_{18}O_{12}$	449.0725
Methyl UroM6	$C_{14}H_{10}O_6$	273.0405
Dimethyl UroM6 glcA	$C_{21}H_{20}O_{12}$	463.0882
Dimethyl UroM6	$C_{15}H_{12}O_6$	287.0561
UroM5 glcA	$C_{19}H_{16}O_{13}$	451.0518
UroM5 sulfate	$C_{13}H_8O_{10}S$	354.9765
UroM5	$C_{13}H_8O_7$	275.0197
Methyl UroM5 glcA	$C_{20}H_{18}O_{13}$	465.0675
Methyl UroM5	$C_{14}H_{10}O_7$	289.0354
Nasutin	$C_{14}H_6O_6$	269.0092
Nasutin glcA	$C_{20}H_{14}O_{12}$	445.0412
EA glcA	$C_{20}H_{14}O_{14}$	477.0311
EA sulfate	$C_{14}H_6O_{11}S$	380.9558
EA	$C_{14}H_6O_8$	300.999
Methyl-EA glcA	$C_{21}H_{16}O_{14}$	491.0467
Methyl-EA sulfate	$C_{15}H_8O_{11}S$	394.9715
Methyl-EA	$C_{15}H_8O_8$	315.0146
Dimethyl-EA glcA	$C_{22}H_{18}O_{14}$	505.0624
Dimethyl-EA sulfate	$C_{16}H_{10}O_{11}S$	408.9871
Dimethyl-EA	$C_{16}H_{10}O_8$	329.0303

Uro = urolithin; glcA = glucuronide; sulfoglcA = sulfoglucuronide; EA = ellagic acid

3.3.10 Quantification of polyphenols in animal diet

All diets for analysis were stored in airtight containers at 4 °C until use. A portion of both diets were double bagged and ground to a powder using a hammer. 2 g of each ground pellet was placed into a separate 50 ml centrifuge tube. A second 2 g sample of 'basic diet' ground pellet was placed into a third 50 ml centrifuge tube where epigallocatechin gallate and ellagic acid were added in methanol at 1.8 mg/g each to match the w:w quantities added to the polyphenol supplemented pellets in production and samples were dried. The pellets were defatted 3 times in hexane. Following drying 10 ml of 5 % formic acid in methanol was added to each sample and these were vortexed and left to mix for 1 hour on a shaker at 4 °C. The 10 ml solution was then removed to a fresh tube and the extraction process was repeated a further 4 times. 1 ml of the sample solution was transferred to HPLC vials. A calibration curve of EGCG and EA were made ranging from 100 µg/ml–1 µg/ml, giving a good coverage of the anticipated polyphenol concentration in the sample. The samples were analysed using a Luna C18 250 x 4.6, 5 µm column on a Agilent 1100/1200 HPLC-DAD-MS system using a gradient of solvent A (0.1 % aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile) with a flow rate of 1 ml/min and a run time of 40 minutes using the gradient program as follows: 0–10 min, 5–17 % B; 10–12 min, 17 % B; 12–17 min, 17–25 % B, 17–25 min, 25–35 % B, 25–30 min, 35–50 % B, 30–35 min, 50–100 % B, 35–40 min 100 % B, 40–41 min 100–5 % B, 41–45 min 5 % B. Recovery of the polyphenols from the extraction was 90 % and 92 % for ellagic acid and EGCG respectively. The polyphenol content as milligram per gram of pellets was calculated following correction for the polyphenol recovery.

3.3.11 Statistical analysis

Data underwent standard model diagnostics to assess assumptions of normality in the residuals, potential outlier detection and influential data points. Where appropriate transformations of the data were undertaken based on box-cox plots to provide a normal distribution.

Body weight, polyphenol content in pellets, VEGF stimulation by injection samples, and pellet consumption data were analysed untransformed. Body weight by

injection type was analysed using ANOVA analysis. Body weight by diet type and pellet consumption data were analysed by t-test. The data for the VEGF stimulation activity in the injection samples and the polyphenol content in pellets were analysed by ANOVA with Tukey multiple-comparison post-hoc test.

ANOVA analysis of the plaque size, circumference and thickness was undertaken with untransformed data, after which a backwards elimination was conducted. A Welch-Satterthwaite t-test on rank transformed data was used to determine significance levels of diet or injection type. The effect of the VEGF/EGCG/EA injection in comparison to the VEGF only injected group was analysed through a Welch-Satterthwaite t-test on rank transformed data.

Transformations of the plasma lipid data were performed as presented in Table 3.4. Backward elimination based on ANOVA analysis was performed and post hoc group differences were assessed using Tukey's honest significant difference (HSD) multiple comparison procedure. The effect of the VEGF/EGCG/EA injection in comparison to the VEGF only injected group was analysed through a Welch-Satterthwaite t-test on rank transformed data.

Table 3.4: A summary of the transformation used to conduct statistical analysis on the data for total cholesterol, LDL cholesterol, triglycerides and calculated HDL cholesterol

Time	Parameter	Transformation
Week 3	Total Chol	Square root
	LDL	Square root
	Trigs	Logarithmic
Week 6	Total Chol	Square root
	LDL	Square root
	Trigs	Logarithmic

3.4 Results

3.4.1 Body weights and daily diet intake of the animals

Body weight and pellet consumption were monitored over the duration of the study for welfare purposes and to monitor if there were any differences based on intervention.

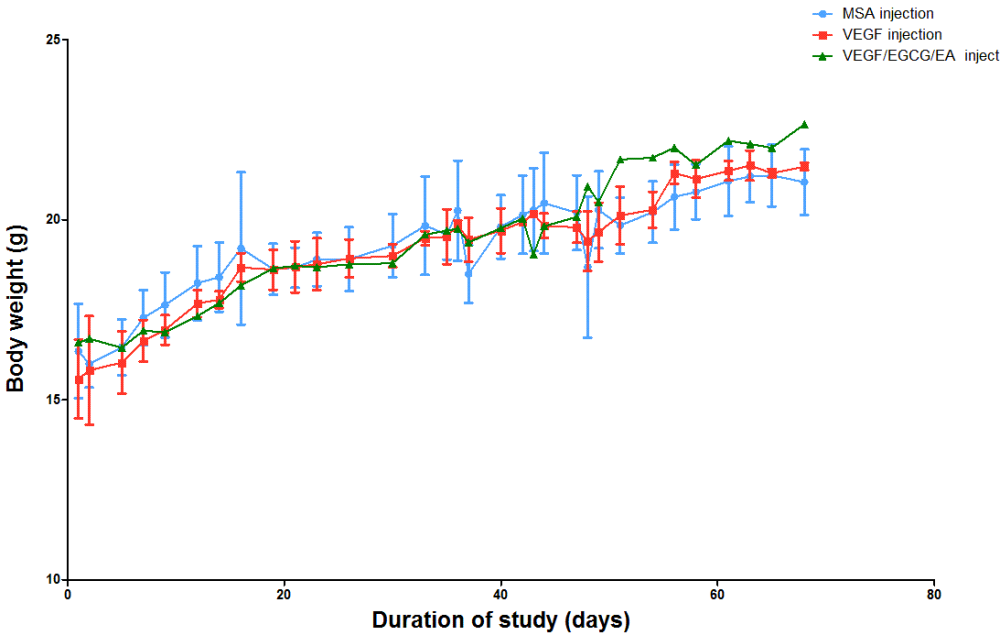
Over the study period there were fluctuations in body weight following the intraperitoneal injections but this was to be expected and the animals regained the weight and continued growth after a couple of days. Neither the injection type (Figure 3.4 A) nor the diet type (Figure 3.4 B) had any significant effect on weight gain in the mice.

There was no significant difference in average pellet consumption per mouse per day (Figure 3.5). The mice fed the 'Basic diet' consumed 2.6 g/mouse/day while the mice fed the 'EGCG/EA diet' consumed 2.4 g/mouse/day.

3.4.2 Quantifying EGCG and EA content of the manufactured pellets

To determine whether any loss of polyphenols in the pellets had occurred through the manufacture process quantification of EGCG and ellagic acid in the manufactured diets was undertaken. The 'Basic diet' contained little to no polyphenol while the 'Basic diet + EGCG/EA', where polyphenols were added prior to extraction, and the 'EGCG/EA diet', manufactured with EGCG and EA, had much higher level of EGCG and EA ($p = <0.001$). There was a small loss of 1 % of EA in the pellets through manufacture but this was not significant (Figure 3.6). However, there was a significant loss of 21.6 % of EGCG in the pellets through manufacture ($p = < 0.001$; Figure 3.7). This equated to the final concentration of 1.78 g/kg ellagic acid and 1.41 g/kg EGCG in the manufactured diet fed to the animals and. This translated to an average daily dose per mouse of 4.3 mg ellagic acid and 3.4 mg EGCG.

A



B

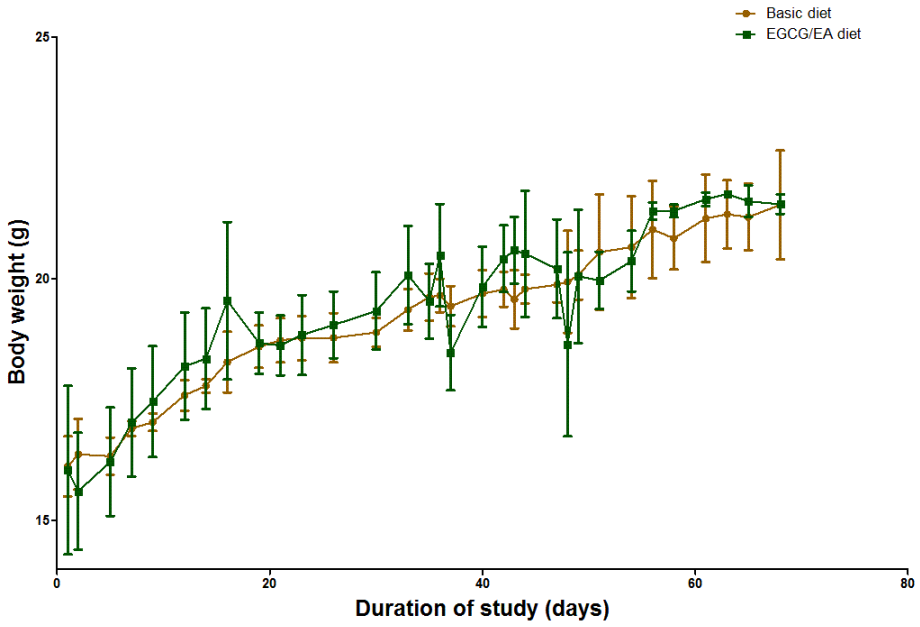


Figure 3.4: Mouse body weight progression during study depending on injection type (A) or diet type (B).

The animals were weighed three times a week and the weights recorded against the day since the start of the intervention per batch. These were plotted into a line graph and the data is presented as mean ± standard deviation. There was no significant difference between either weights dependent on injection type or diet type; n = 17 - 35.

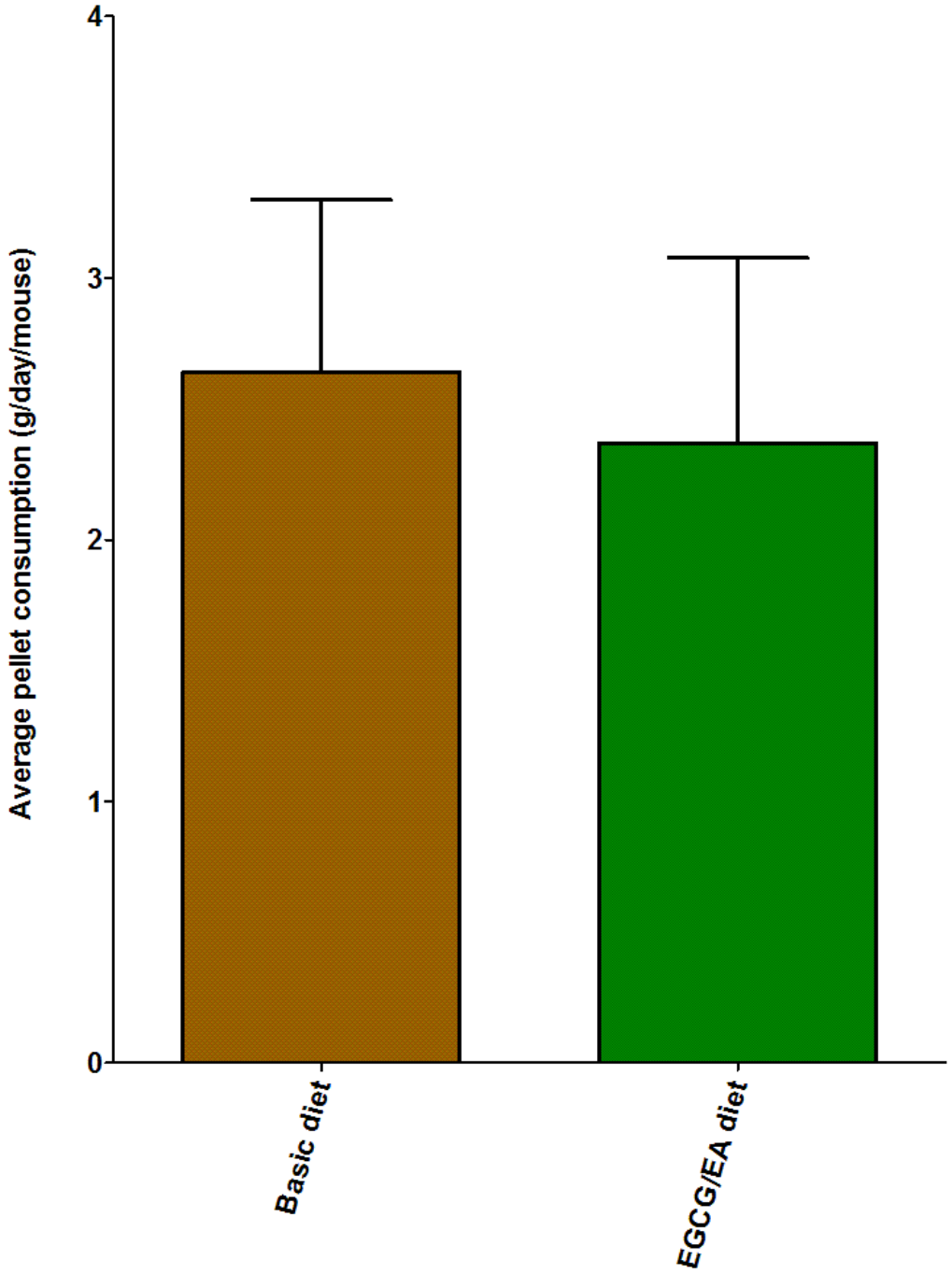


Figure 3.5: The average daily pellet consumption per mouse of the two experimental diets.

The average pellet consumption per mouse in grams was calculated for both studies across the duration of the study. The graph shows the average of these values across all cages and batches for each diet type ± standard deviation. There was no significant difference between the daily pellet consumption of the two experimental diets; n = 35 - 52.

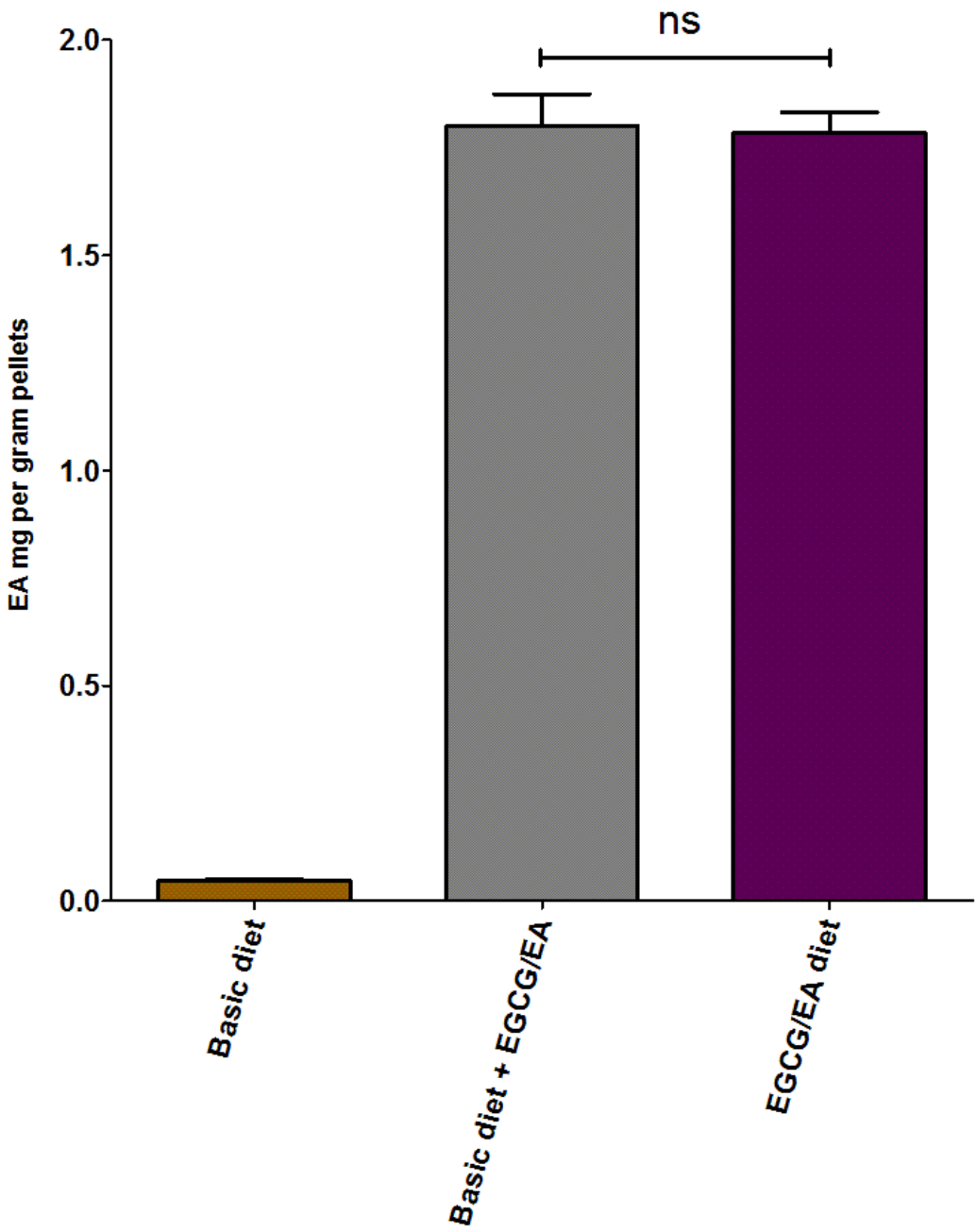


Figure 3.6: Ellagic acid content in the manufactured experimental diets

The ellagic acid content the manufactured diet in mg/g powdered pellets was determined after methanol extraction by HPLC/DAD/LCMS. The 'Basic pellets' supplemented with EGCG and EA to a 1.8 g/kg dose were spiked immediately prior to extraction for comparison. The data is presented as mean ± standard deviation after correction for loss during extraction process. The 'Basic diet' did not contain significant amounts of polyphenols. There was no significant difference in the ellagic acid content of the 'EGCG/EA diet', manufactured with EGCG and EA to a 1.8 g/kg dose, compared to the 'Basic pellet + EGCG/EA' which were supplemented with 1.8 g/kg polyphenols immediately prior to extraction; n = 3.

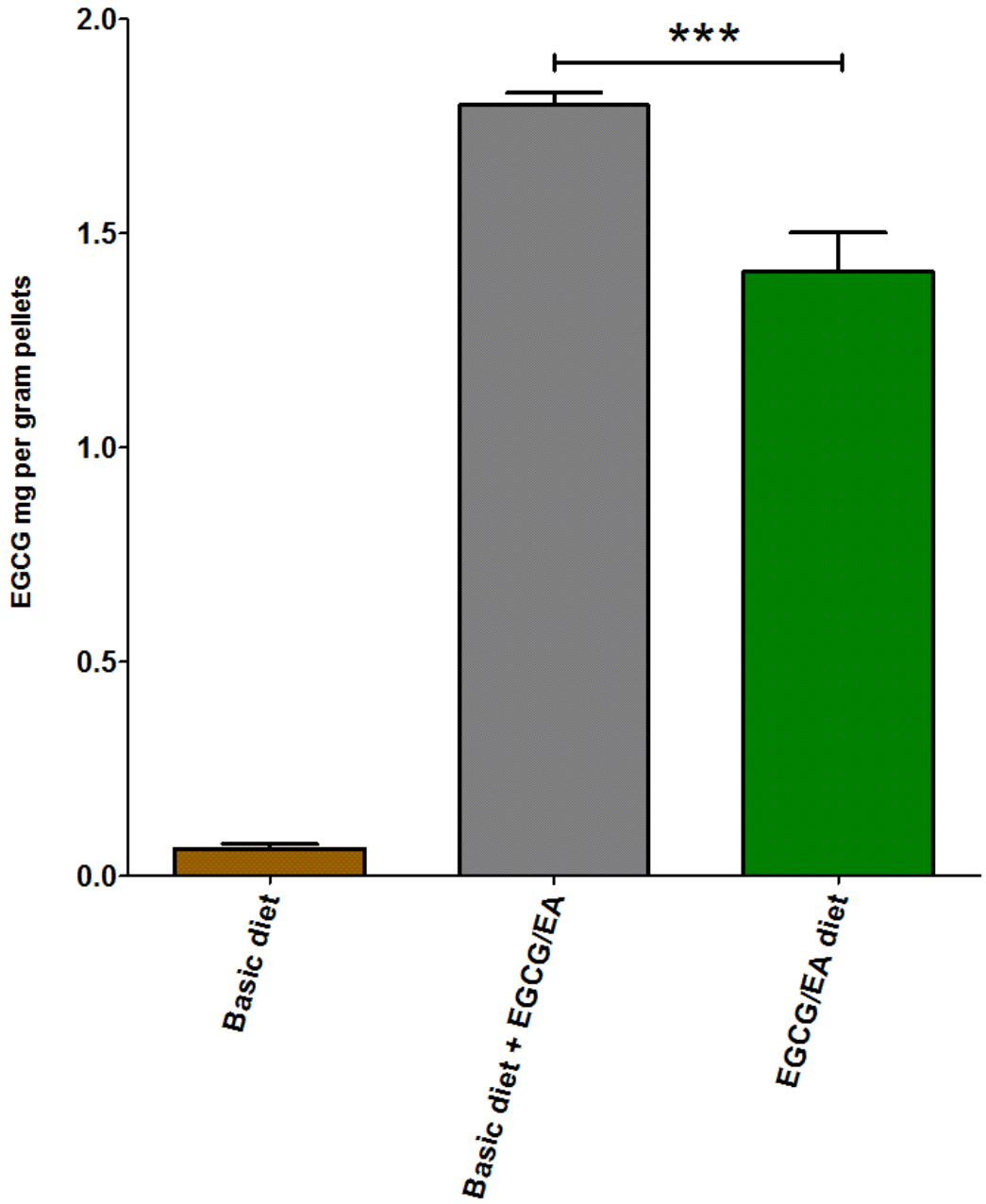


Figure 3.7: EGCG content in the manufactured experimental diets

The EGCG content the manufactured diet in mg/g powdered pellets was determined after methanol extraction by HPLC/DAD/LCMS. The 'Basic pellets' supplemented with EGCG and EA to a 1.8 g/kg dose were spiked immediately prior to extraction for comparison. The data is presented as mean ± standard deviation after correction for loss during extraction process. The 'Basic diet' did not contain significant amounts of polyphenols. There was a significant difference between the EGCG content in the 'EGCG/EA diet', manufactured with EGCG and EA to a 1.8 g/kg dose, compared to the 'Basic pellet + EGCG/EA' which were supplemented with 1.8 g/kg polyphenols immediately prior to extraction.(*** $p = \leq 0.001$). This equated to a 21.6 % loss of EGCG through manufacture; n = 3.

3.4.3 Effect of injection solutions on VEGFR-2 phosphorylation of HUVECs

The injection samples from the animal study were used to treat HUVECS to determine whether the VEGF in the sample was able to stimulated VEGFR-2 and therefore active upon delivery to the animal. All VEGF injection samples were able to significantly stimulate VEGFR-2 phosphorylation in HUVECs (see Figure 3.8**Error! Reference source not found.**). Although there were fluctuations in the VEGF stimulatory activity of each individual VEGF injection sample, treatment of HUVECs with the VEGF injection samples stimulated, on average, 85 % VEGFR-2 phosphorylation in comparison to a freshly defrosted VEGF control ($p = \leq 0.001$). Limited to no VEGFR-2 phosphorylation was seen in any of the MSA or VEGF+EA+EGCG injection samples.

3.4.4 Quantification of atherosclerotic plaque in aortic sinus following intervention

The degree of atherosclerotic plaque development in the aortic sinus of the animals was measured in 3 ways. 1) Atherosclerotic plaque area, presented as both the percentage and μm^2; 2) Atherosclerotic plaque circumferential expansion, presented as both the percentage and µm; and 3) the maximal plaque thickness at the thickest portion of plaque, presented in µm (Figure 3.9-Figure 3.13).

There were no significant differences in plaque area and plaque circumference between any of the treatment groups ($p = > 0.05$). The maximal plaque thickness was significantly lower in the 'basic diet + VEGF/EGCG/EA inj.' group in comparison to the 'Basic diet + VEGF inj.' group ($p = 0.0213$). There were no significant differences in maximal plaque thickness between any of the other groups ($p = > 0.05$). The data for all treatments at each time point was pooled to determine whether the common factor, the high fat and high cholesterol diet, was able to induce disease over study duration. There was a significant increase in plaque area, circumference and thickness between baseline and 3 weeks post first injection, and between 3 weeks and 6 weeks post first injection, showing progression of atherosclerosis between each time point ($p = 0.001 – 0.0001$; Figure 3.14).

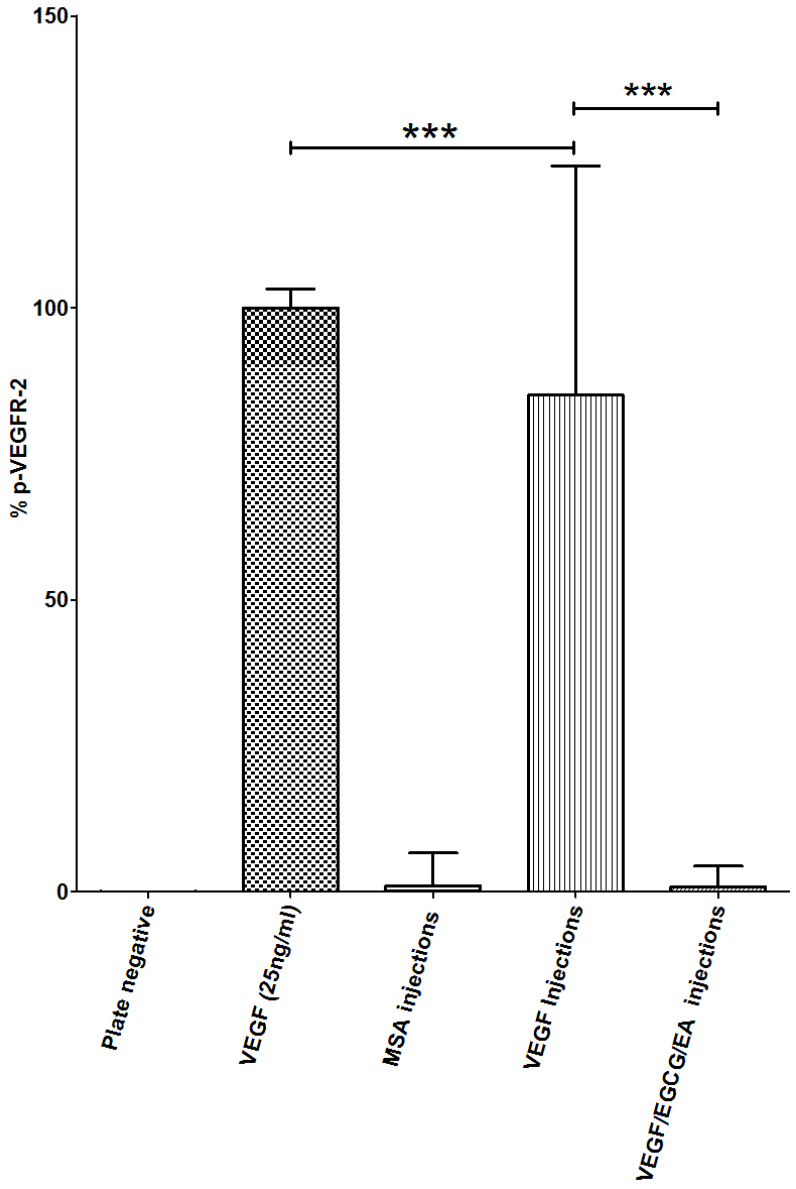


Figure 3.8: The VEGFR-2 phosphorylation in HUVECs stimulated with the VEGF, MSA and VEGF/PP injections diluted to 25 ng/ml.

A sample of the injection solution used in the animal study was snap frozen in dry ice at time of injection. The injection solutions were diluted to a 25 ng/ml VEGF concentration in basal media and used to stimulate HUVECs for 5 minutes. A VEGF positive treatment made from a freshly defrosted aliquot was used as a comparator. The activity of the injections samples is expressed as a percentage of the VEGFR-2 phosphorylation in comparison to this VEGF positive control. Results are plotted as mean ± standard deviation The VEGFR-2 stimulating activity of the VEGF injection samples was significantly lower than the freshly defrosted VEGF samples. Limited to no stimulation was observed by the MSA or VEGF+EA+EGCG injection samples. There was no significant difference between the VEGFR-2 phosphorylation activities of the MSA injected samples versus the VEGF+EA+EGCG injected samples. *** $p = \leq 0.001$; $n = 44 – 105$.

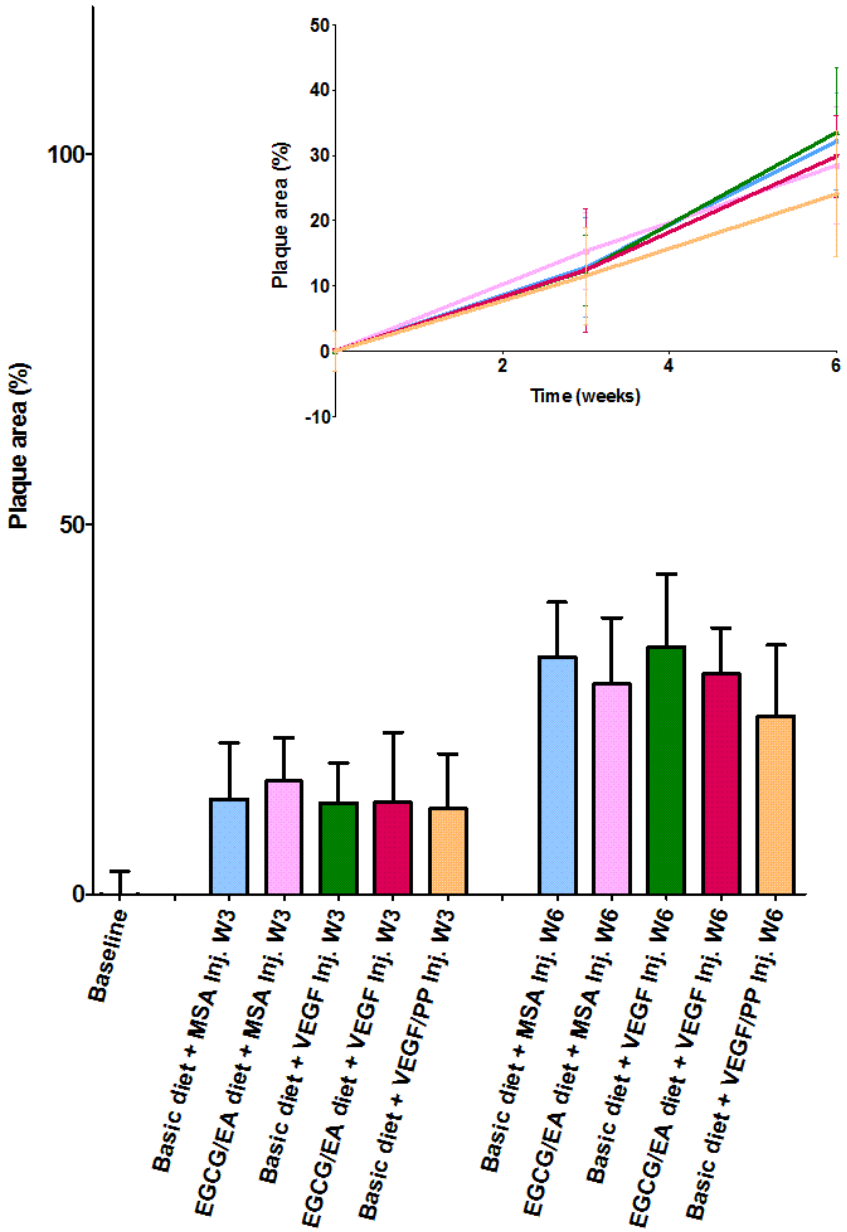


Figure 3.9: The atherosclerotic plaque area as a percentage of the total area of the lumen at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent atherosclerotic plaque percentage after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation of the percentage plaque area to lumen area. The inset graph presents the same data as a progression over time. There was no significant difference between any of the treatment at each of the time points; n = 8-9.

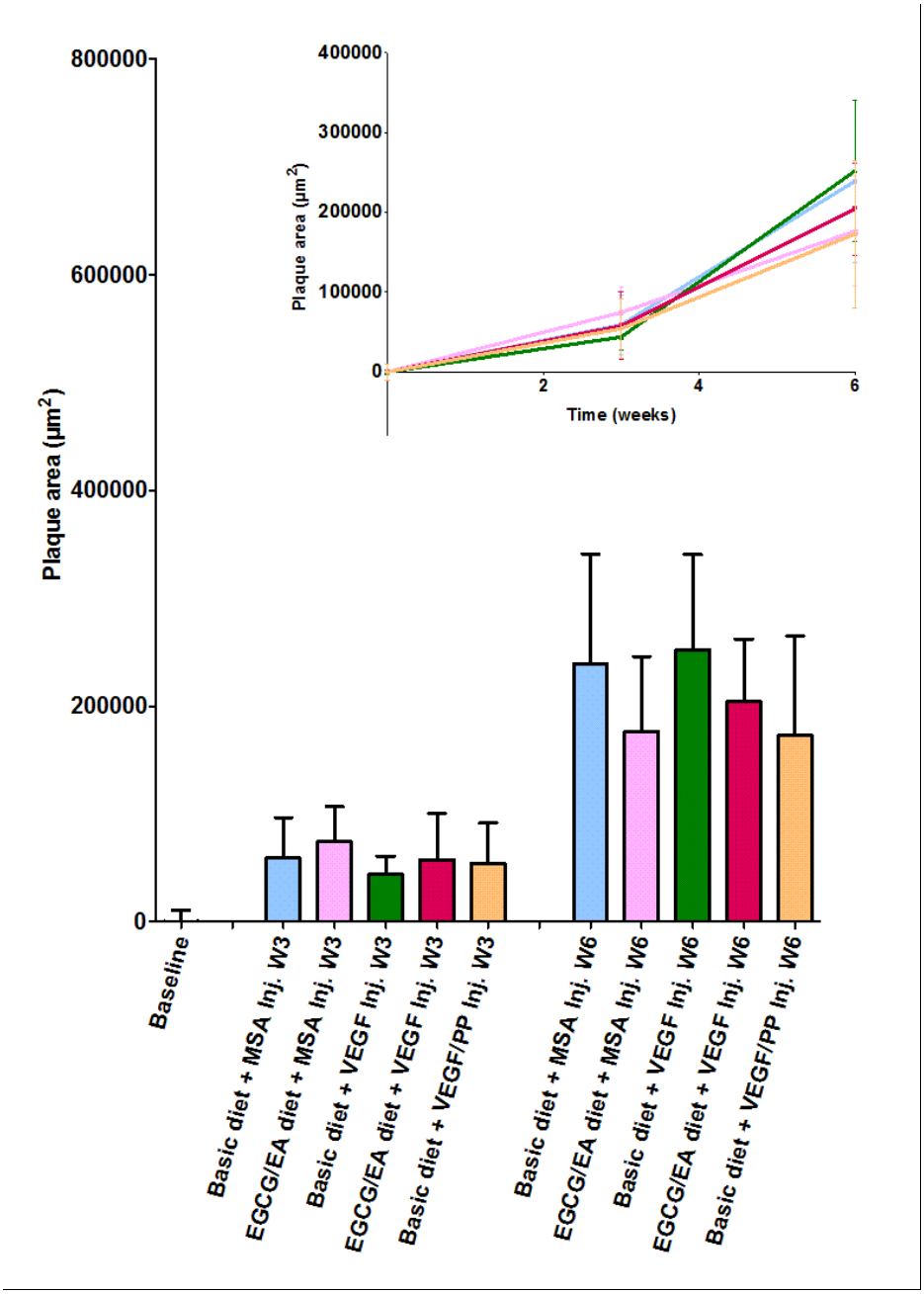


Figure 3.10: The atherosclerotic plaque area in µm2 at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent atherosclerotic plaque area (μm^2) after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. There was no significant difference between any of the treatment at each of the time points; n = 8-9.

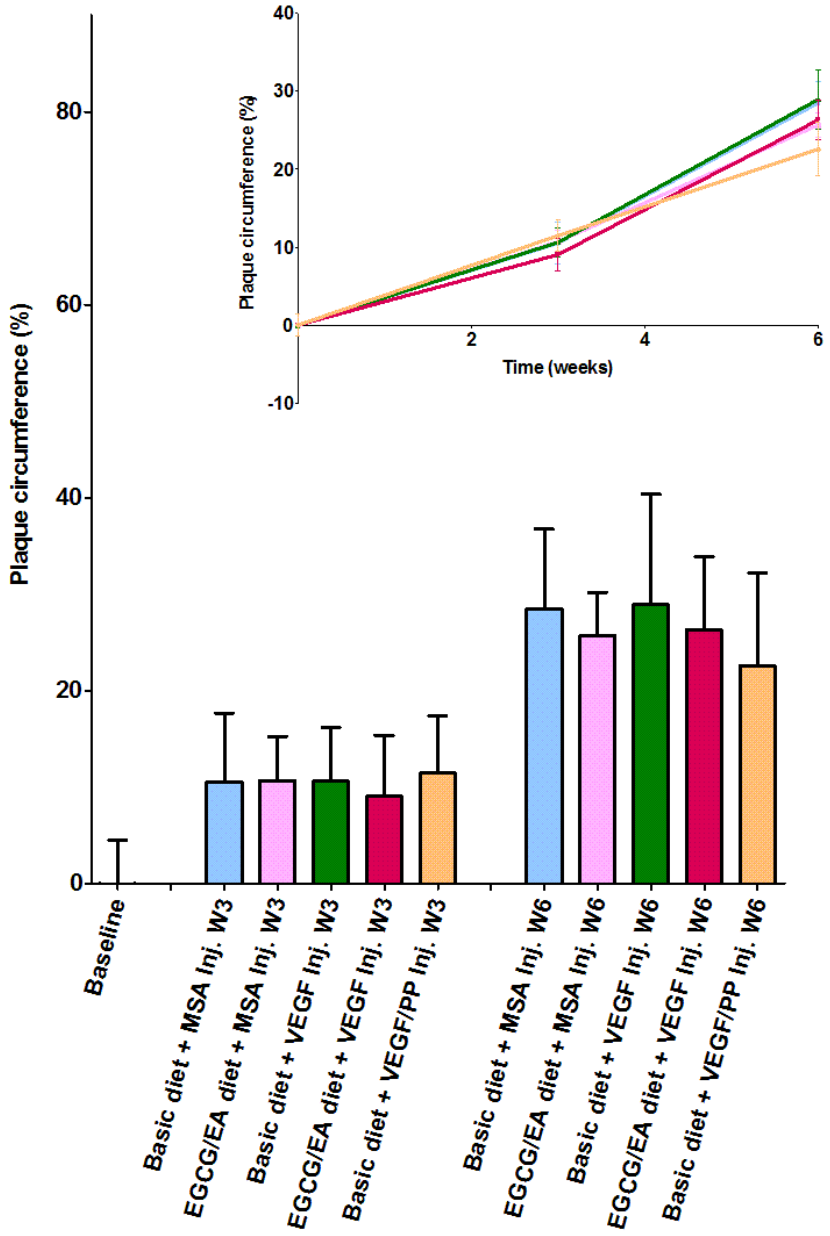


Figure 3.11: The atherosclerotic plaque circumferential expansion as a percentage of the total lumen circumference at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent percentage of atherosclerotic plaque circumferential expansion after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. There was no significant difference between any of the treatment at each of the time points; n = 8-9.

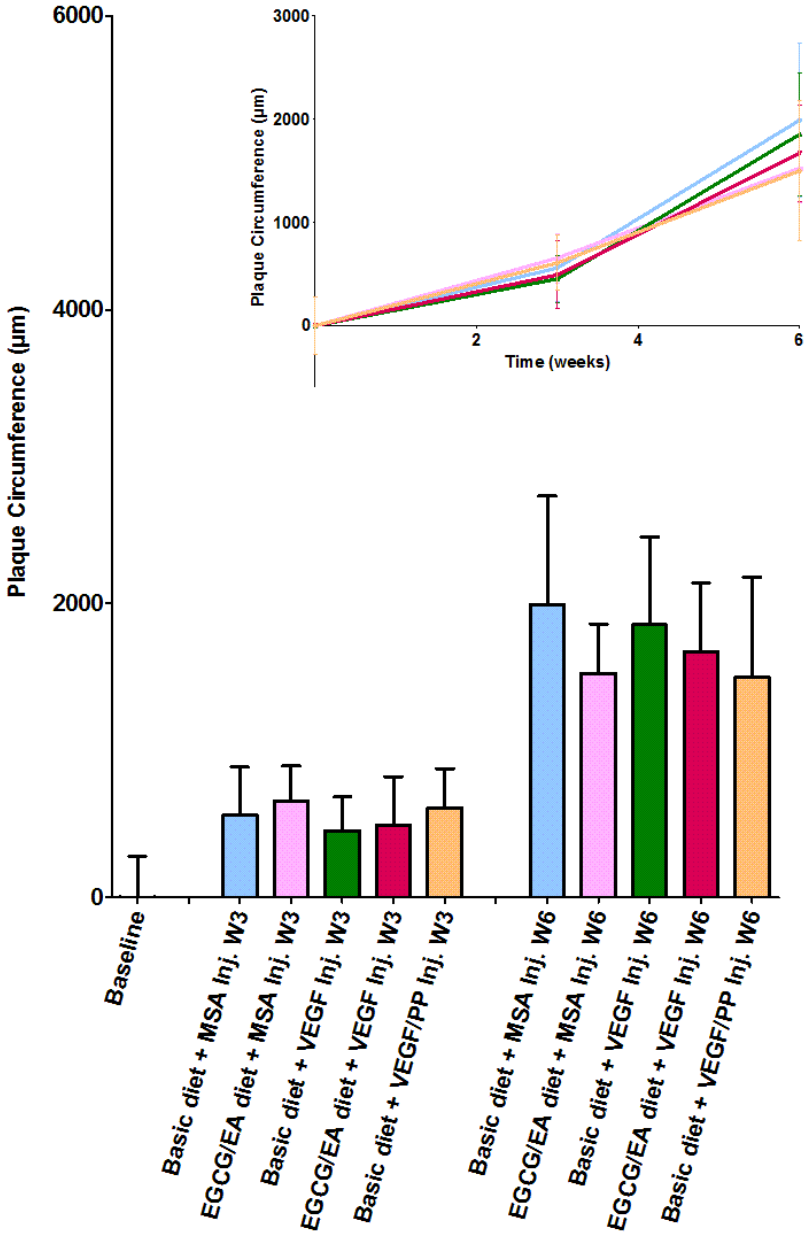


Figure 3.12: The atherosclerotic plaque circumferential expansion in µm at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent atherosclerotic plaque circumferential expansion (µm) after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. There was no significant difference between any of the treatment at each of the time points; n = 8-9.

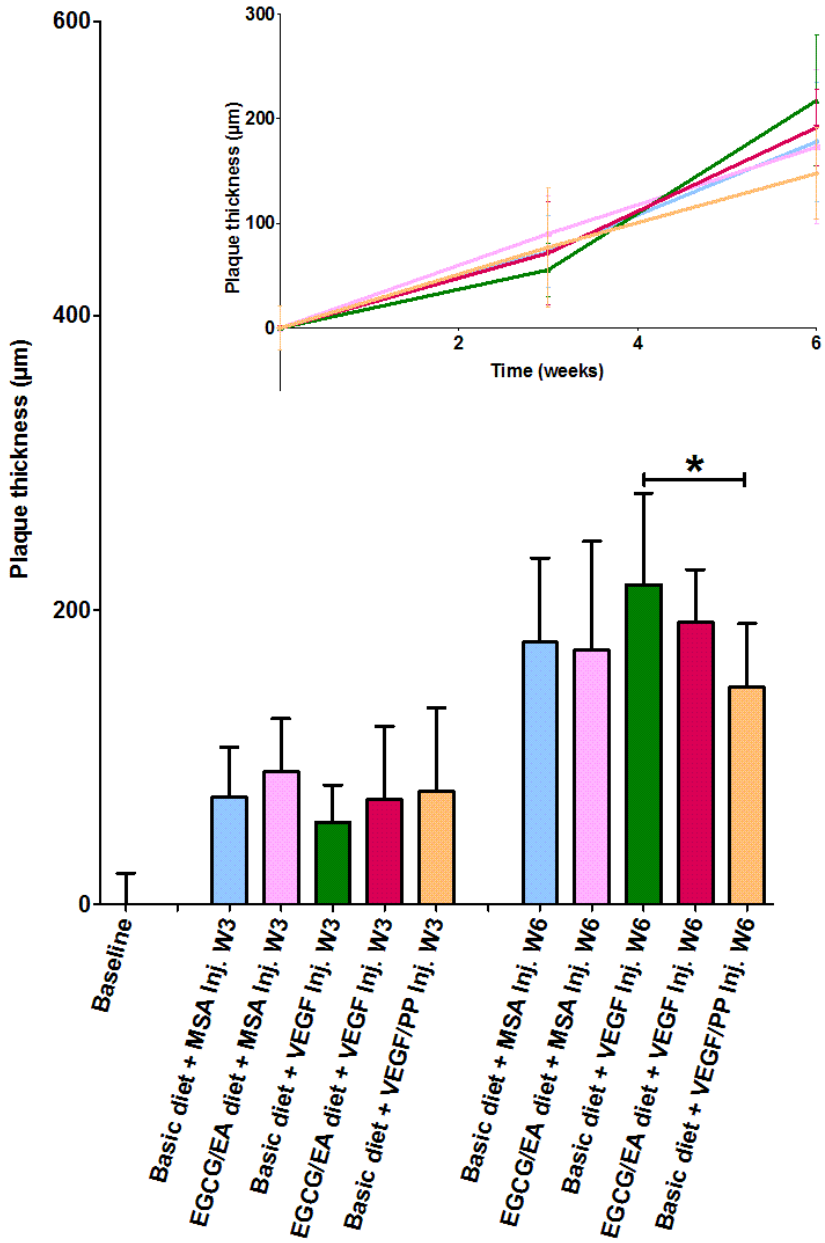


Figure 3.13: The atherosclerotic plaque maximal thickness in µm at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent atherosclerotic plaque maximal thickness (µm) after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. At week 3 there was no significant difference between any of the treatments. At week 6 the 'Basic diet + VEGF/PP inj.' was significantly lower than the 'Basic diet + VEGF inj.' group (* p = 0.0213). There were no significant differences between any other groups at the week 6 time point; n = 8-9.

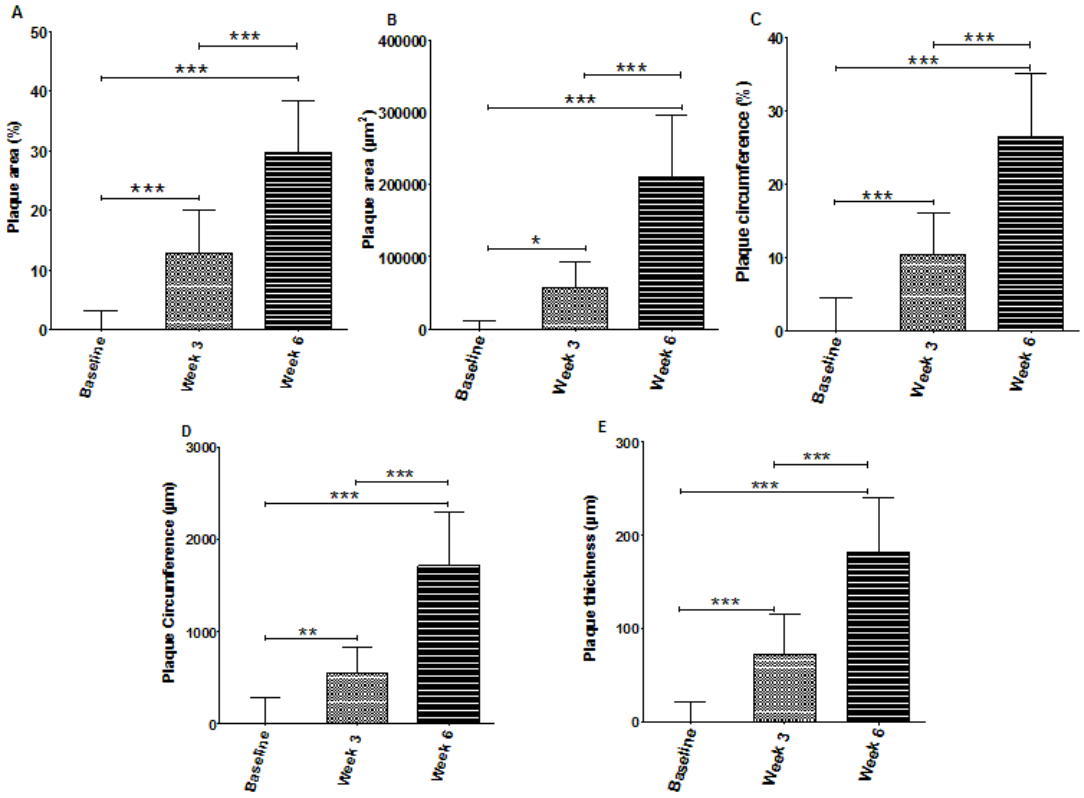


Figure 3.14: The atherosclerotic plaque area, plaque circumference, and maximal plaque thickness for all mice at baseline, week 3 and week 6 post first injection.

All animals were aged 8 weeks at baseline and had undergone 4 weeks induction of atherosclerosis with an atherogenic diet. Animals continued on the atherogenic diet until the end of the study and at week 3 and week 6 the plaque in the aortic sinus of a subset of animals was measured. Atherosclerotic plaque was measured as plaque area in % (A) and μm^2 (B), plaque circumferential expansion in % (C) and µm (D), and maximal plaque thickness in µm (E). The data is presented as the mean ± standard deviation. In all measurements there was a significant increase between baseline and week 3 and between week 3 and week 6 (** p = <0.01; *** p = <0.001); n = 10 - 44.

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3.4.5 Quantification of plasma lipids

Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were quantified on the Randox Daytona^{+} biochemical analyser using the manufacturer's protocol.

The values for total cholesterol ranged from 31.6 - 65.7 mmol/L (Figure 3.15) while the values for LDL cholesterol ranged from 5.55 - 20.46 mmol/L (Figure 3.16) across the study. When comparing the values at 3 weeks post first injection the 'EGCG/EA diet + MSA inj.' groups had significantly higher total cholesterol and LDL cholesterol values than all other groups ($p \leq 0.05$). At 6 weeks post first injection a raised total cholesterol ($p = 0003$) and LDL cholesterol ($p = 0.0001$) was significantly associated with the EGCG and EA supplemented 'EGCG/EA diet', regardless of injection type. The MSA injection, was also significantly associated with an increased LDL cholesterol in comparison to the VEGF injected animals ($p = 0.0054$). HDL cholesterol was below the limit of detection in all samples and could therefore not be quantified.

Triglyceride levels in the plasma were also significantly reduced at 3 weeks post first injection in the 'Basic diet + VEGF inj.' treatment in comparison to all other treatments ($p = \leq 0.05$). Furthermore, at 3 weeks post first injection there was a significantly higher triglyceride level in the plasma from the 'Basic diet + VEGF/PP inj.' treated mice in comparison to the plasma triglyceride levels in the 'Basic diet + VEGF inj.' group ($p = 0.0009$). At 6 weeks post first injection there was no significant difference between the treatment groups in plasma triglyceride levels (Figure 3.17).

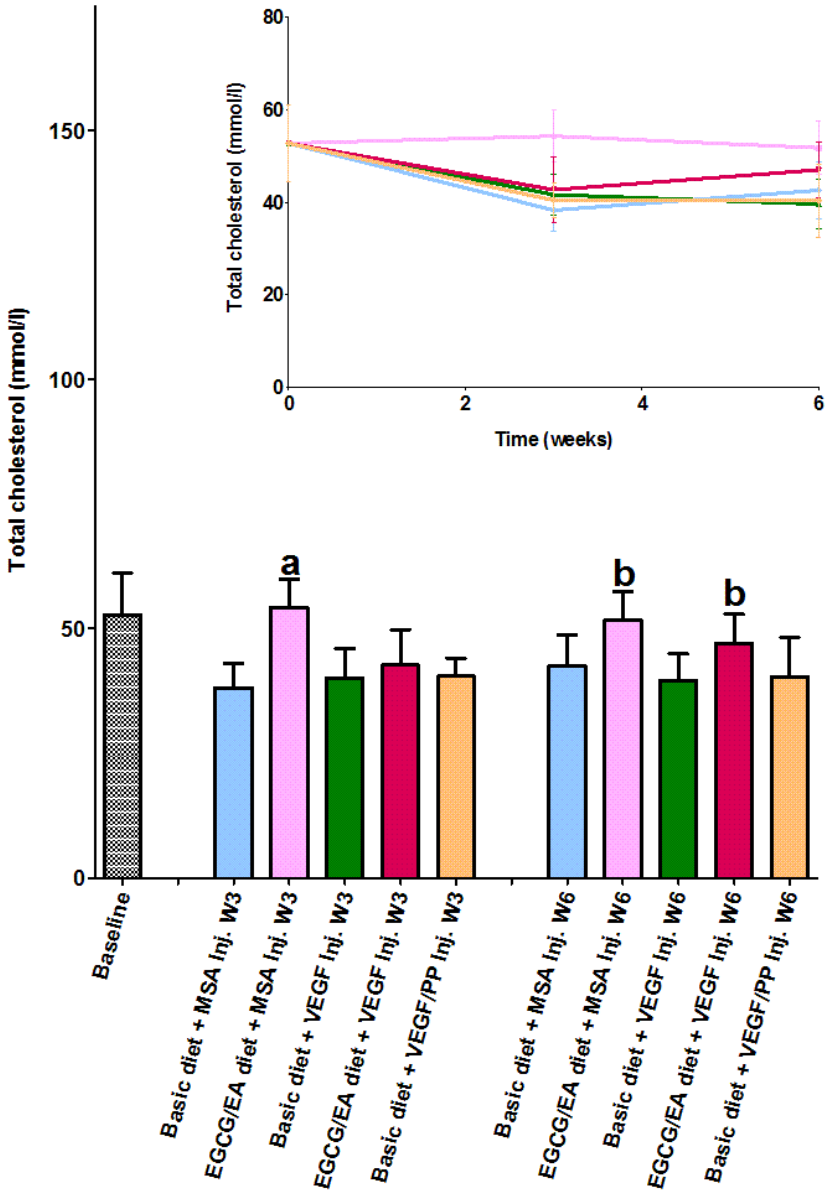

Figure 3.15: The plasma total cholesterol levels in mmol/l at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent plasma total cholesterol levels after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. At week 3 post first injection 'EGCG/EA diet + MSA inj.' treatment was associated with raised total cholesterol ($p = \leq 0.05$) in comparison to all other groups (**a**). At week 6 post first injection there was a significant association between higher total cholesterol levels and the PP diet in general, irrelevant of injection type ($p = 0.0003$) (**b**); n = 8-9.

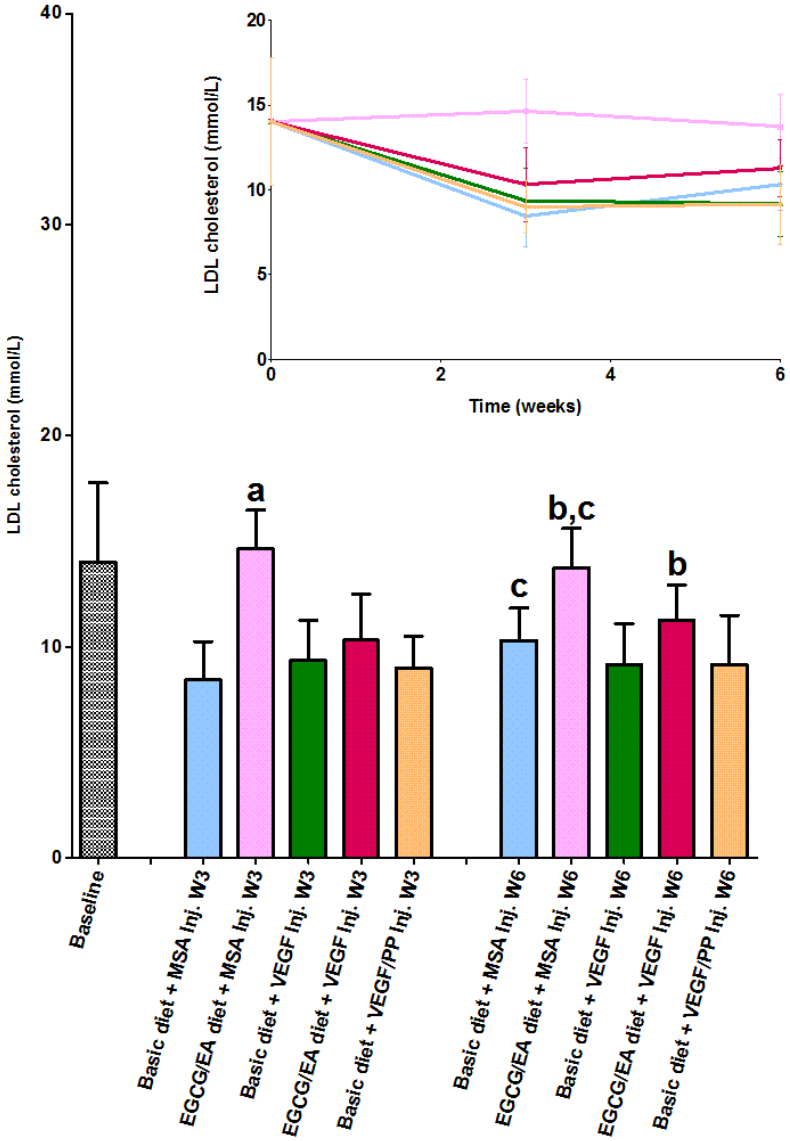

Figure 3.16: The plasma LDL cholesterol levels in mmol/l at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent plasma LDL cholesterol levels after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. At 3 weeks post first injection the 'EGCG/EA Diet + MSA inj.' treatment was associated with raised plasma LDL cholesterol ($p = \leq 0.05$) in comparison to all other groups at this time point (**a**). At 6 weeks post first injection there was a significant association between higher LDL cholesterol levels and the PP diet in general, irrelevant of injection type ($p = 0.0003$) (**b**) and LDL cholesterol and the MSA injection, irrelevant of diet type ($p = 0.0054$) (**c**); n = 8-9.

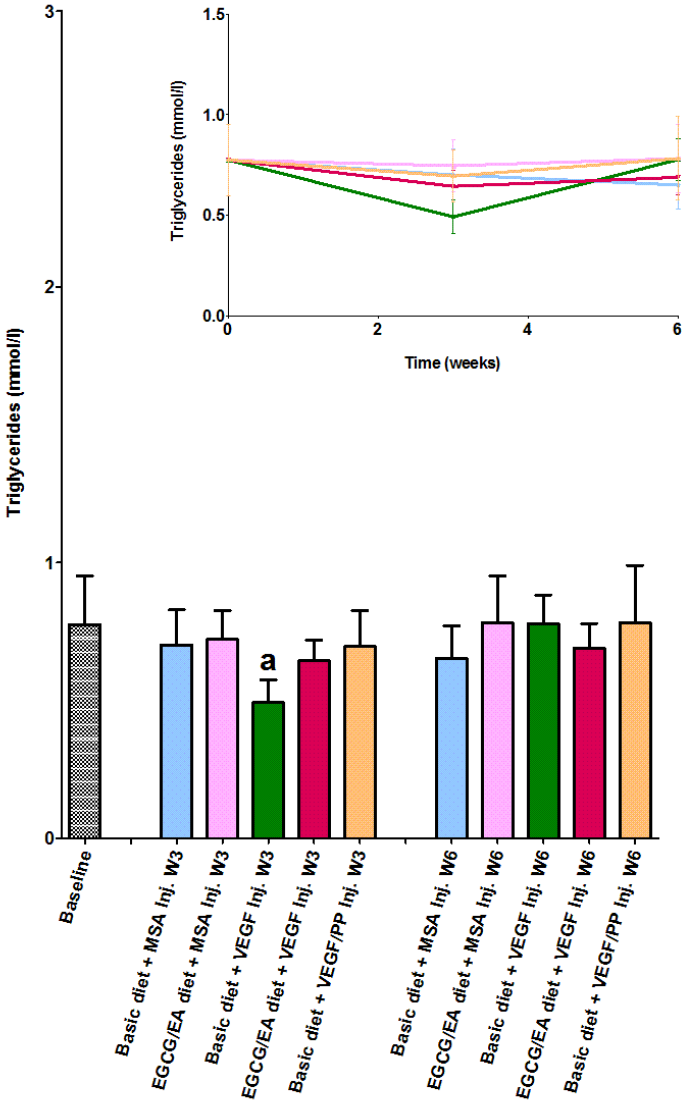

Figure 3.17: The plasma triglyceride levels in mmol/l at baseline, week 3 and week 6 post first injections.

All animals underwent a 4 week an atherogenic diet and the baseline measurement represent plasma triglyceride levels after this 4 week induction. At baseline, 3 weekly injections were undertaken and, where required, the animals received an EGCG and EA supplemented diet ('EGCG/EA diet'). The data is presented as the mean ± standard deviation. The inset graph presents the same data as a progression over time. At 3 weeks post first injection the 'Basic diet + VEGF inj.' treatment was associated with lower triglyceride levels ($p = \leq 0.05$) in comparison to all other groups at this time point (**a**). At week 6 there were no significant differences in plasma triglycerides levels between any treatments; n = 8-9.

3.4.6 Quantification of polyphenols in the plasma

The quantification and evaluation of polyphenols in the plasma from the mice was performed using a published method (García-Villalba et al., 2016). No EGCG derived compounds identified in Table 3.2 were found in any sample, including the quality control samples spiked with known concentrations of EGCG and EA, demonstrating that the method was not appropriate for analysis of compounds associated with EGCG consumption in plasma. It was therefore impossible to determine the plasma concentration of EGCG and its metabolites. In all the plasma samples from mice fed the 'Basic diet' no polyphenol metabolites could be detected. Similarly, no polyphenols or metabolites were detected in the plasma samples from mice in the 'Basic diet + VEGF/EGCG/EA injection' treatment group. However, in the plasma samples from mice fed the 'EGCG/EA diet' there were detectable amounts of a variety of the EA derived compounds presented in Table 3.3. Where standards were available the compounds were quantified against calibration curves to give an accurate concentration. A summary of the compounds quantified using standards can be found in Table 3.5. Quantification of the compounds where a standard was not available was undertaken by comparing the relative areas to the relative area of the known internal standard, chrysin. A summary of these compounds can be seen in Table 3.6. It is worth noting that ellagic acid extraction from the QC samples was poor, with only 20 % of spiked concentration being quantified in the QC samples.

Table 3.5 Table demonstrating all EA metabolites found at quantifiable concentrations in the plasma of the mice fed an EGCG and EA supplemented diet and their concentration in nmol/L as quantified by HPLC/QTOF-MS.

	Ellagic acid	Uro A-3 glucuronide	Uro A sulfate
Retention time (min)	7.25	6.43	8.11
Conc. (nmol/L)	12.9	19.5	15.3

Table 3.6: Table demonstrating all EA metabolites found in the plasma of the mice fed an EGCG and EA supplemented diet and their relative areas in comparison to a chrysin standard as measured by HPLC/QTOF-MS.

	Methyl-EA	Methyl-EA glucuronide	Dimethyl -EA	Dimethyl-EA glucuronide	Uro-A sulfoglucuronide	Uro-C sulfate	Uro-C glucuronide	Uro-C sulfoglucuronide	Uro-C sulfoglucuronide
Retention time (min)	9.82	6.91	12.37	7.77	4.68	7.76	6.29	4.76	5.28
Relative area	4.5	2.5	35.5	67.8	10.8	152.9	92.4	41.4	157.0

3.5 Discussion

The aim of this chapter was to determine whether polyphenols which are capable of potent *in vitro* inhibition of VEGF induced VEGFR-2 phosphorylation could inhibit VEGF induced atherosclerosis. The study reported in this chapter was designed to investigate a number of hypotheses. 1) VEGF injection will induce significant atherosclerosis above a mouse serum albumin (MSA) injected control; 2) Dietary supplementation with EGCG and ellagic acid, potent *in vitro* inhibitors of VEGF-induced VEGFR-2 phosphorylation, will inhibit VEGF-induced atherosclerosis; 3) dietary supplementation with EGCG and EA will be able to limit endogenous VEGF signalling associated with atherosclerotic progression; 4) mixing EGCG and EA with VEGF prior to intraperitoneal injection will completely ablate any VEGF stimulation of atherosclerosis.

It has previously been demonstrated that intraperitoneal injection of VEGF in both a high cholesterol fed New Zealand white rabbit and ApoE-/-ApoB^{100}-/- genetically modified mouse model was able to significantly stimulate atherosclerotic plaque growth (Celletti et al., 2001a, Celletti et al., 2001b). This suggests that the induction of atherosclerotic disease by VEGF was not dependent on genetic hypercholesterolemia but that cholesterol induction of the disease may be important. However, the work presented in this chapter has shown that intraperitoneal injection of VEGF into ApoE-/- mice fed a high fat, high cholesterol did not induce atherosclerosis, at least under these study conditions.

3.5.1 Was the VEGF active upon injection?

To determine whether the VEGF in the injections was active at time of injection the VEGFR-2 phosphorylation capacity of the injections was monitored throughout the study. All VEGF injections were active at the time of injection while the MSA and VEGF/PP injections were not. It should be noted that, on average, only 85 % of the VEGF stimulatory capacity was retained in the VEGF injection samples in comparison to a freshly made VEGF control. However, no blood was taken from the mice immediately after injection so we cannot conclusively say whether the VEGF reached the blood stream. Limited data is present on the capacity for proteins to pass into the blood stream following intraperitoneal injection although a study in 2014

investigated the distribution of fluorescent labelled mouse serum albumin after different routes of injection. Intraperitoneal injection resulted in limited distribution at 1 hr and systemic distribution at 3 hour post-injection (Kijanka et al., 2014). Given that the *in vivo* VEGF half-life has been shown to be around 30 minutes (Eppler et al., 2002), perhaps a delayed systemic distribution limited the effective VEGF dose in circulation. However, the injection protocol used in this chapter was identical to that used in previous studies where strong induction of atherosclerosis was observed following intraperitoneal injection of VEGF (Celletti et al., 2001b). This suggests that the dissemination of active VEGF after injection is unlikely to be the cause of the lack of atherosclerosis induction observed in this chapter. Furthermore, there were two additional VEGF injections undertaken in the discussed study in comparison to previous reports providing two more opportunities for VEGF induction of the disease. Considering the VEGF was active at time of injection, the similarity of injection protocol to previous studies where VEGF induced atherosclerosis, and the repeated number of injections to previous reports it can be concluded that the lack of disease induction by VEGF it unlikely to be due to inactive or non-functioning injections. However, the study design may be improved through use of intravenous injection of the VEGF utilising a VEGF expressing adenovirus as previously reported (Heinonen et al., 2013).

3.5.2 Did cholesterol induction impact the result?

The study presented in this chapter used the ApoE knockout mouse as a model. This differs from the ApoE-/-ApoB^{100}-/- used by Celletti et al. The lack of Apolipoprotein E in the animal leads to a reduced ability to clear LDL and vLDL cholesterol (Meir and Leitersdorf, 2004), leading to high circulating cholesterol levels. The additional mutation, ApoB^{100}-/- mice used in the Celletti et al. paper resulted in the animals expressing only the Apolipoprotein B48 truncated protein which is incapable of binding to the LDL receptor, and therefore clearing the cholesterol it carries, inducing further hypercholesterolemia. However, this anticipated difference in cholesterol burden between the ApoE-/- and the ApoE-/-ApoB^{100}-/- models was overcome by inclusion of an atherogenic diet (AIN57BB, TestDiet) which increased the cholesterol dose from 0.25 % used in the Celletti et al. study to 1.25 %. The

atherogenic diet did induce atherosclerosis over time and the atherosclerotic plaque sizes achieved were similar or slightly larger than to those observed in other studies which fed ApoE-/- mice a similar atherogenic diet for a similar duration (Huang et al., 2015, Cao et al., 2009).

3.5.3 Was the plaque susceptible to angiogenesis induction?

Considering angiogenesis plays a role in progressing plaque growth and not initiating it (Khurana et al., 2004) it could be suggested that in mice with a smaller level of plaque (≤100 µm thickness (Doyle and Caplice, 2007)) diffusion of the molecules can occur freely across the tissue without the need for induction of microvessel growth, which a supplementation of VEGF would aid. The average value for maximal plaque thickness at baseline, and therefore at the time of the first injection in the presented study was 61.5 µm, which is within a distance where diffusion should not be limited. This provides one explanation as to why the supplementation of VEGF did not induce atherosclerosis as VEGF supplementation may not provide the same advantage to atherosclerotic plaque growth in this tissue as it would if there was a need for microvessel growth. Unfortunately, the data provided in the Celletti et al. paper (Celletti et al., 2001b) only reports a normalised to zero baseline maximal plaque thickness therefore a comparison cannot be drawn. However, if we compare the plaque areas of the control serum albumin sham-injected mice between the study presented in this chapter and the Celletti et al. a comparison between the model induction of atherosclerosis can be undertaken. In the study presented in this chapter the mice in the 'basic diet + MSA injection' treatment arm at 3 weeks post first injection had an average plaque area of 79,547 μm^2 while Celletti et al. reported an average plaque area of 64,238 μm^2 for the serum albumin injected mice at 3 weeks post first injection. This slightly larger amount of plaque in the sham-injected mice in the study presented in this chapter suggests that induction of baseline atherosclerosis was unlikely to be an issue, as the controls in both studies developed similar plaque levels.

3.5.4 Impact of EGCG dietary intake on atherosclerosis

Another interesting result from this study was the demonstration that EGCG and EA supplementation to the diet does not reduce atherosclerotic plaque size, irrelevant

of injection type. A number of studies demonstrated that green tea extract, of which EGCG is the major component, has anti-atherosclerotic properties with up to around 35 % reduction in plaque size (Miura et al., 2001, Minatti et al., 2012, Tijburg et al., 1997, Hong et al., 2014). ApoE-/- mice fed with EGCG in their water at 0.8 g/L (estimated 4.8 mg/day (Bachmanov et al., 2002)) for 7 weeks up to the age of 15 weeks, a similar duration of study and EGCG dose to the 3.4 mg/day presented in this chapter. A reduction in atherosclerotic development was observed which was attributed to reduced vascular endothelial induced Notch signalling (Yin et al., 2016), a known VEGF associated signalling pathway (Jakobsson et al., 2009). Considering a loss of EGCG was observed during the manufacture of the pellets it is possible that the stability of EGCG may have played a part in the lack of effect in the discussed study. The importance of pH, temperature, humidity and oxygen on EGCG degradation has been demonstrated previously (Ananingsih et al., 2013) and perhaps the experimental cage conditions reduced the EGCG content of the pellets through degradation. However, fresh pellets were provided 3 times a week and in a previous report it was shown that green tea powder retained up to 95 % of the green tea catechins over 112 days of storage under similar conditions to those in the cage (25 °C and 58 % humidity) suggesting that stability of EGCG in the pellets is unlikely to be the reason for the lack of observed effect on plaque size reported in this chapter (Li et al., 2011).

All studies to date which have demonstrated an atherosclerosis inhibitory effect of either green tea extract or pure EGCG have had the extract of polyphenol provided in the drinking water or through intraperitoneal injection (Chyu et al., 2004, Miura et al., 2001, Yin et al., 2016). It has previously been shown in humans that the bioavailability of EGCG was affected when the dose was given alongside food, in comparison to water alone (Naumovski et al., 2015). This may explain why these studies observed an anti-atherosclerotic effect of EGCG while no effect was observed in the discussed study where dietary delivery of EGCG was undertaken. Unfortunately, we cannot determine whether free EGCG was able to reach the plasma of mice in the discussed study due to instability of EGCG during the polyphenol plasma extraction procedure, evidenced by the lack of EGCG in quality

control samples. Previously, daily injection of 10 mg/kg body weight for 6 weeks reduced cuff-induced atherosclerosis by 73 % in comparison to the control group (Chyu et al., 2004). In the study described in this chapter, if some free polyphenol had been provided in the 'basic diet + VEGF/EA/EGCG injection' group it will be below the EGCG dose of 36 µg/kg provided in the injection due to the VEGF/polyphenol interactions reducing the free EGCG available. In the 'basic diet + VEGF/EA/EGCG injection' treatment arm the maximal plaque thickness was significantly reduced in comparison to the VEGF only injected animals ($p = 0.0213$). This suggests that intraperitoneal delivery of EGCG could limit the progression of atherosclerosis however all other measurements of plaque size for this treatment group were not significant therefore the result of reduced plaque thickness should be treated with caution. The difference between the previously observed inhibitory effects of intraperitoneal EGCG injection on plaque size to the data presented in this chapter is likely due to the concentration of EGCG used.

3.5.5 Impact of EA dietary intake on atherosclerosis

In the presented study the mice were fed with an EA 0.18 % supplemented diet. A previous study in rats, albeit with a much higher dose of EA (2 % diet supplementation), found reduced intimal thickening and lipid deposition with intimal thickness measuring less than 100 µm in EA fed mice. The data presented in this chapter demonstrate the maximal plaque thickness in the mice fed an EGCG and EA supplemented diet at 3 and 6 weeks post diet change far exceeds this at an average of 140 µm and 241 µm respectively. Given that rats have a higher food intake than mice this would equate to a much high EA daily dose which may suggest that a higher ellagic acid dose is required for plaque reduction, although drawing comparisons between rats and mice is not ideal (Rani et al., 2013). Similarly after 1 % EA diet supplementation in rabbits only 2 % of the intima contained lipids compared to 43 % in the control group showing that strong anti-atherosclerotic effects have been observed in two different animal models of disease with a higher EA diet dose (Yu et al., 2004). Ellagic acid was detected in the plasma of mice albeit at very low levels (12.9 nmol/L). Mice were fed *ad libitum* up until time of death and the concentration of the polyphenols in the plasma will depend on the length of time

since the mice last ate which was not able to be monitored. However, the amount of ellagic acid quantified in the quality control samples was lower than the spiked dose suggesting the plasma concentration of ellagic acid is likely to be underestimated. Ellagic acid is known to be poorly soluble (Bala et al., 2005) and this may explain this reduced concentration in the quality control samples. If we assume the overall loss of ellagic acid through the extraction process is the same as with the quality control samples the average ellagic acid content in plasma would be 645 nmol/L, which is in a similar range to previous reports in other species (Hamad et al., 2009, Murugan et al., 2009, Lei et al., 2003). The analysis of plasma samples presented in this study does indicate that ellagic acid and the urolithins are present in plasma following ellagic acid consumption. Furthermore, urolithins are present in higher concentrations than ellagic acid, while the majority of metabolites appear as phase-II conjugates. The amount and type of urolithins produced following EA consumption has been reported to be dependent on microbiota (Tomás-Barberán et al., 2014), specifically *Gordonibacter* Spp (Romo-Vaquero et al., 2015). Since urolithin B was not detected in any of the mouse plasma samples reported in this thesis, the microbiota of the mice appears to be more similar to the human urolithin A producer phenotype rather than the human urolithin B/isourolithin A producer phenotype (Tomás-Barberán et al., 2014). Interestingly, in a recent pomegranate intervention only participants with the urolithin B producer phenotype were reported to be susceptible to the lipid lowering effects of the pomegranate intervention (Gonzalez-Sarrias et al., 2016). However, given that ellagic acid and its metabolites were detected in the plasma, and that a higher ellagic acid dose previously demonstrating anti-atherosclerotic effects, it is likely that one reason for a lack of observed anti-atherosclerotic effect by ellagic acid consumption in this chapter is due to the lower dose of ellagic acid given to the mice.

3.5.6 Could plaque vulnerability be altered by VEGF injection?

While the physical amount of atherosclerosis was not changed by VEGF injection or dietary supplementation with EA and EGCG, the existing literature evidence suggests that the dangerous nature of atherosclerosis is the vulnerability of the plaque, as rupture and thrombus formation pose more of a risk than a large but stable plaque

(Eliasziw et al., 1994). Increased angiogenesis is a feature of vulnerable plaques, with the suggestion that the vulnerable vessel formation may be associated with growth factors such as VEGF (Chen et al., 1999, Juan-Babot et al., 2003, Fleiner et al., 2004). While the size of the plaque can give an indication of disease level, perhaps plaque morphology and vulnerability may be more critical in evaluating the role of VEGF injection in atherosclerotic disease. In the presented study measurements to investigate plaque vulnerability were not undertaken due to time restrictions but further staining for morphology with histological methods such as the Masson's trichrome or Movat's pentachrome stains to determine incorporation of elastin, collagen and fibrinogen (Virmani et al., 2000, Virmani et al., 2006) would be beneficial. Equally, von Willebrands factor (Virmani et al., 2005), VEGF (Chen et al., 1999) or VEGFR-2 staining could provide insight into microvessel density of the plaque and angiogenic potential of the disease based on treatment type.

3.5.7 Impact of treatments on plasma lipid levels

The levels of plasma cholesterol observed in the study were very high but similar to previously reported data. In the presented study total cholesterol values ranged from 31.6 – 65.7 mmol/L (or 1222- 2541 mg/dl). Plasma cholesterol levels in ApoE-/- mice fed a western type diet (0.25 % cholesterol) have been reported up to 2000 mg/dL (Getz and Reardon, 2006). Given that the cholesterol load from the diet was higher for the discussed study it was expected the total cholesterol and LDL cholesterol values exceeded this upper measurement of 2000 mg/dL. It was reported in this chapter that HDL could not be quantified in the plasma which matches previous observations that ApoE-/- mice fed an atherogenic diet have undetectable levels of HDL cholesterol, even compared to a chow diet fed ApoE-/- mouse (Zhang et al., 1994). At 3 weeks post first injection total cholesterol ($p = 0.0022$) and LDL cholesterol ($p = 0.0005$) were significantly higher in the PP diet + MSA injection group compared to all other treatment groups. A similar pattern was observed at 6 weeks post first injection with the PP diet, irrelevant of injection type, causing an increase in total cholesterol ($p = 0.0003$) and LDL cholesterol ($p = 0.0001$). This is in contrast to previous data which demonstrates either no effect of EGCG or EA on plasma cholesterol (Chyu et al., 2004, Miura et al., 2001, Ding et al., 2014) or, in the

case of ellagic acid, a reducing effect on plasma cholesterol levels (Park and Kang, 2012) in the ApoE-/- model. In other animal models EGCG has also demonstrated lipid lowering activities in high fat models (Chen et al., 2011b, Raederstorff et al., 2003). There were no studies which demonstrated an induction effect of EA on plasma cholesterol although 100 mg/day oral gavage with green tea extract in the LDL receptor double knockout mouse model resulted in raised total cholesterol levels, although lower triglyceride levels, in comparison to controls (Minatti et al., 2012). The increase in plasma cholesterol in the EGCG and EA fed mice was not due to increased pellet consumption as the average consumption of the 'EGCG/EA diet' per mouse per day was not significantly different (2.4 g) to the 'basic diet' (2.6 g). Hepatotoxicity of EGCG has been previously reported following high doses in mice (Lambert et al., 2010), with the lethal dose of EGCG for hepatocytes *in vitro* being 200 µM (Galati et al., 2006). Liver damage can result in the accumulation of LDL cholesterol (Ooi et al., 2005) and could explain the raised cholesterol level if this were the case. However previous hepatotoxicity studies of EGCG report a much higher dose than used in this study (over 500 mg twice daily) is required before hepatotoxic markers are observed (Lambert et al., 2010). Conversely, it has been reported that ellagic acid has hepatoprotective effects *in vivo* (Singh et al., 1999, Girish et al., 2009) suggesting that the results observed in this chapter are not due to hepatotoxic effects of the polyphenols.

Another interesting observation is that the 'diet + VEGF injection' group had significantly lower plasma triglyceride levels at 3 weeks post injection when compared to the other groups ($P = < 0.05$) and the VEGF injected mice, irrelevant of diet type, had reduced plasma LDL cholesterol levels compared to the MSA injected groups ($p = 0.0054$) at 6 weeks post first injection. This suggests that VEGF injection may be associated with lipid reducing activities. Previously, plasma cholesterol were shown to be raised following injection of a VEGF-A expressing adenovirus into the ApoE-/- mouse (Heinonen et al., 2013). However, in female participants in the SAPHIR study it was reported that increases in VEGF reduced circulating LDL cholesterol levels (Sandhofer et al., 2009). Given the impact that raised LDL cholesterol (Jenkins et al., 1978) and triglycerides (Tkáč et al., 1997) can have on

atherosclerotic development the observation that VEGF injected mice had lower levels of triglycerides and LDL cholesterol can provide an explanation why VEGF injection did not induce atherosclerosis in the discussed study. Triglyceride levels were also raised in the 'basic diet + VEGF/EGCG/EA injection' group in comparison to VEGF injected mice at 3 weeks post first injection ($p = 0.0009$) however this observation was not demonstrated at week 6 therefore these differences in triglyceride levels may be due to other factors, for example postprandial triglyceride elevation (Ueshima et al., 2005).

Finally, it should also be noted that all plasma cholesterol and triglyceride levels, irrelevant of the treatment group, were never significantly raised above the plasma cholesterol and triglyceride levels at baseline. Therefore, conclusions about any of the treatments increasing plasma lipid levels should be treated with caution.

3.6 Conclusions

The study described in this chapter aimed to determine whether EGCG and EA, two potent inhibitor of VEGF-induced VEGFR-2 phosphorylation *in vitro*, are able to inhibit VEGF-induced atherosclerosis *in vivo*.

The data in this chapter demonstrated that intraperitoneal VEGF injection does not induce atherosclerosis in the ApoE-/- mouse model fed an atherogenic diet. However, it does not exclude the possibility that other methods of VEGF delivery or high systemic VEGF levels would not have a pro-atherogenic effect or that VEGF injection may have caused a more unstable, and therefore dangerous, plaque. These are interesting avenues for future investigation. A daily dose of 3.4 mg EGCG and 4.3 mg ellagic acid was not able to reduce atherosclerosis progression in either the VEGF or MSA injected animals. This is likely due to the low dose used in this study in comparison to other reports. Given VEGF was unable to induce atherosclerosis the effect of EGCG and EA on the VEGF in the injection could not be assessed however inclusion of EGCG and EA in the injection did result in reduced maximal plaque thickness after 6 weeks, although not in overall plaque size reduction. Furthermore, EGCG and EA dietary supplementation caused small but significant increases in

plasma total cholesterol and LDL cholesterol levels in contradiction to previous reports.

In summary, even though EGCG and EA were potent inhibitors of VEGF-induced VEGFR-2 phosphorylation *in vitro*, this did not translate to a reduction in atherosclerotic progression in ApoE-/- mice fed an atherogenic diet. However, considering the upregulation of endogenous VEGF associated with atherosclerosis and the atherosclerotic plaque tissue this observation is surprising. To further investigate this chapter 4 will investigate whether serum proteins can affect the interaction of VEGF with EGCG and ellagic acid.

Chapter 4

Serum proteins abolish the strong inhibitory effect of EGCG and EA on HUVEC VEGF signalling

4.1 Abstract

In chapter 2 both ellagic acid and EGCG were identified as potent inhibitors of VEGF-induced VEGFR-2 activation. However, chapter 3 reports that ellagic acid and EGCG dietary supplementation did not affect the atherosclerotic plaque size in $ApoE^{-/-}$ mice. Where EGCG and EA were co-injected with VEGF a small reduction in plaque thickness was seen however the other measurement of plaque size were unaffected. While VEGF-injection did not increase plaque size it was expected that endogenous VEGF upregulation would have occurred within the growing plaques and that the polyphenols would have the potential to limit atherosclerosis progression by acting on endogenous VEGF upregulation. Therefore, the lack of atherosclerosis inhibition by EGCG and ellagic acid was further investigated *in vitro*.

The ability of EGCG to inhibit VEGF-mediated angiogenesis was assessed in the mouse aortic ring assay, where serum is required for tissue growth. Subsequently, the effect of FCS presence in the media on the ability of EGCG and EA, independently and in combination, to inhibit VEGF-induced VEGFR-2 in HUVECs was assessed at the standard 5 minute treatment time and over a longer incubation period. EGCG treatment did not inhibit VEGF-induced angiogenic vessel sprouting in the aortic ring assay compared to VEGF-only treated rings. Inhibition of VEGF-induced VEGFR-2 phosphorylation by ellagic acid and EGCG, independently and in combination, was completely abolished when FCS was present in the media prior to polyphenol addition. Furthermore, it was shown that the VEGFR-2 stimulation activity of VEGF which was rendered completely inactive by pre-mixing with ellagic acid and EGCG in serum-free medium was recovered over time after serum was added to the VEGF/polyphenol mixture.

The presence of FCS has the ability to cause loss of EGCG and ellagic acid inhibition of VEGF-induced VEGFR-2 phosphorylation. Where the FCS is present prior to the addition of the polyphenol complete loss of inhibition is seen. Therefore, in a serum protein rich environment, such as the blood, EGCG and ellagic acid are unlikely to be able to inhibit rapid VEGF-induced VEGFR-2 activation, providing one explanation for the lack of observed effects in the animal study in chapter 3.

4.2 Introduction

In the previous chapter it was shown that under the study model conditions used the VEGF injection was unable to significantly induce higher levels of atherosclerotic disease in the aortic arch of ApoE-/- mice and that the green tea and pomegranate polyphenols, epigallocatechin gallate (EGCG) and ellagic acid (EA), were ineffective at inhibiting atherosclerosis, even in the sham (MSA) injected mice. Given the previous literature discussed in chapter 1 which reports that endogenous VEGF upregulation occurs from the early stages of atherosclerotic plaque build-up, EGCG and EA had the potential to inhibit this endogenous VEGF signalling through interacting with the VEGF ligand to limit atherosclerotic plaque growth. This chapter aims to contribute to the interpretation of the lack of observed atherosclerosis inhibition in the animal intervention carried out in chapter 3 by investigating the impact of inhibitory polyphenols on angiogenesis and the effect of serum proteins on the potent inhibition of VEGF stimulated VEGFR-2 phosphorylation by EGCG and EA *in vitro.*

4.2.1 Polyphenol inhibition of VEGF signalling

As discussed previously inhibition of VEGF signalling has been reported by a wide range of polyphenols via a number of mechanisms (Guruvayoorappan and Kuttan, 2007, Oh et al., 2010, Pratheeshkumar et al., 2012, Luo et al., 2012, Luo et al., 2009, Dann et al., 2009, Oak et al., 2006, Subramanian and Arul, 2012, Osada et al., 2004, Lee et al., 2004). Early reports into the effects of polyphenols on VEGF signalling had demonstrated polyphenols could inhibit VEGFR-2 phosphorylation in both unstimulated and VEGF stimulated cells at supra-physiological concentrations (Lee et al., 2004, Labrecque et al., 2005, Lamy et al., 2006, Lamy et al., 2002, Teller et al., 2009, Pratheeshkumar et al., 2012, Neuhaus et al., 2004, Pan et al., 2011, Shimizu et al., 2010). The literature also demonstrates that EGCG was able to limit the amount of tagged VEGF ligand bound to immobilised VEGFR-2 (Kondo et al., 2002) while ellagic acid, through *in silico* modelling, was predicted to bind to VEGFR-2 at the ATP binding site of the receptor limiting VEGFR-2 phosphorylation (Wang et al., 2012). More recently it has been demonstrated that potent inhibition of VEGFR-2 phosphorylation by is possible by EGCG and EA is achievable at low nanomolar

concentrations (IC50 88 and 230 nM respectively) (Cerezo et al., 2015, Moyle et al., 2015). Similar results were replicated in chapter 2 of this thesis with IC50 values of 96 and 310 nM respectively for EGCG and EA inhibition of VEGF-induced VEGFR-2 phosphorylation after pre-mixing the VEGF and polyphenols. Furthermore, the pomegranate derived polyphenol punicalagin and the microbial metabolites urolithin D and urolithin C were shown to potently inhibit VEGF-induced VEGFR-2 phosphorylation at relatively low concentrations of 49nM, 0.2µM and 3.7 µM respectively. It was also demonstrated that all the polyphenols tested inhibited VEGF-induced VEGFR-2 phosphorylation primarily by interacting with the VEGF ligand itself. This supported a previous observation by Moyle et al. who reported that the rapid inhibitory action of EGCG on VEGF-induced VEGFR-2 phosphorylation was through strong binding of EGCG to the VEGF ligand itself, based on experiments where dialysis of the VEGF/PP treatment solution did not result in recovery of VEGF activity (Moyle et al., 2015). Additionally, it was reported that the strength of inhibition of VEGFR-2 phosphorylation by all polyphenols in this model correlated to the predicted binding affinities of the polyphenols to VEGF estimated through *in silico* analysis (Cerezo et al., 2015). Overall, the data thus far suggests that the method of rapid inhibition of VEGF signalling by polyphenols at low concentrations is due to interactions of the polyphenol with the VEGF ligand, predicted to occur at the VEGFR-2 binding regions of the VEGF dimer.

4.2.2 Polyphenol inhibition of VEGF induced angiogenesis

There are a number of models used to investigate the effects of polyphenols on angiogenesis and VEGF-induced angiogenesis. VEGF-induced vascular endothelial cell tube formation has been shown to be inhibited by polyphenols including the green tea catechins (Pan et al., 2011, Lamy et al., 2006, Lin et al., 2003, Liu et al., 2008, Tang et al., 2003, Pratheeshkumar et al., 2012). Treatment with the green tea catechins was undertaken at a high dose (100 µM) and it was seen that EGCG and ECG were most effective at this dose in comparison to EGC and EC suggesting the importance of the galloyl group in this activity (Kondo et al., 2002). In the same model 2.5 µM ellagic acid was reported to inhibit VEGF-induced tube formation as well as this inhibition being identified as dose-dependent (Wang et al., 2012). The

aortic ring assay models aortic angiogenesis. Polyphenol rich extracts (Lu et al., 2010, Wen et al., 2008, Kang et al., 2013, Daleprane et al., 2012) as well as pure polyphenols (Chen and S. Easton, 2011, Wang et al., 2012, Pratheeshkumar et al., 2012, Guruvayoorappan and Kuttan, 2007, Matsubara et al., 2005) were able to inhibit new vessel generation from *ex vivo a*ortic tissue. Ellagic acid in serum free media was able to inhibit 70 % new vessel generation after 48-72h treatment at a 5µM concentration (Wang et al., 2012) however EGCG has yet to be investigated in this model. In the chick chorioallantoic membrane once again a range of polyphenols were able to inhibit this angiogenesis (Pan et al., 2011, Pratheeshkumar et al., 2012, Oh et al., 2010, Hseu et al., 2011, Favot et al., 2003) including ellagic acid which was able to inhibit 90 % of VEGF-stimulated angiogenesis in this model at 10 µM concentration (Wang et al., 2012). In the *in vivo* matrigel plug assay of angiogenesis cell invasion into the plug was prevented by EGCG inclusion at 220 or 440 µM concentrations after 7 days. However, the vessel density or branch points were not quantified so the anti-angiogenic potential was not measured (Singh et al., 2002). Numerous other polyphenols have demonstrated inhibition of blood vessel generation in this model (Lamy et al., 2008, Lamy et al., 2006, Pratheeshkumar et al., 2012) as well as limiting pathological angiogenesis within a cancer cell and polyphenol laden matrigel plug (Lu et al., 2010, Chakraborty et al., 2008, Luo et al., 2009). Mice which had undergone daily intaperitoneal injections of 50 mg/kg ellagic acid for 25 days had a reduced tumour volume following implantation of a plug containing an adenocarcinoma cell line, although the haemoglobin content as a marker of angiogenesis was not measured in this study (Wang et al., 2012). Overall the data thus far suggests that the polyphenols which have demonstrated VEGF signalling inhibition may also have the capacity to inhibit VEGF-induced angiogenesis in both simple an complex models. Furthermore, the potent and rapid biologically relevant inhibitors of VEGF-induced VEGFR-2 phosphorylation, EGCG and ellagic acid, have both previously demonstrated anti-angiogenic effects

4.2.3 Interaction between polyphenols and proteins

It has been known for many years that polyphenols are capable of readily interacting with proteins. The role of polyphenol and protein interactions was first discussed in relevance to the bioavailability and bioactivity of dietary derived compounds for example, the impact of milk addition on tea or cocoa polyphenol bioavailability and anti-oxidant capacity. The majority of polyphenol-protein interactions are non-covalent although the binding affinities do tend to be associated with similar structural patterns (Cao et al., 2016, Plet et al., 2015) Conversely, quercetin has been shown to selectively and covalently bind human serum albumin in the presence of peroxides (Kaldas et al., 2005). However, over the last 5 years there has been a boom in published papers discussing the interactions between a wide variety of polyphenols and biologically relevant proteins. To date, a wide range of polyphenol compounds have been investigated for their interaction with blood proteins such as albumin and haemoglobin although the green tea catechins, perhaps because of their strong association with cardiovascular improvements, have been most widely investigated. Epicatechin exhibited relatively low binding constants in comparison to the other green tea catechins although polymerisation of the epicatechin into procyanidin increased the binding to BSA (Watrelot et al., 2015). The presence of a galloyl moiety in the 3' position of the C ring of green tea catechins inferred up to 100 times greater binding affinity (Minoda et al., 2010). In a comparison of binding constants epicatechin gallate (ECG) and EGCG were among some of the polyphenols with the strongest binding to BSA reported to date (Zinellu et al., 2014) although it should be noted that not all polyphenols have been investigated. In another study ellagic acid had a reasonable binding constant (8.47 10^4 L/Mol) and therefore affinity for human serum albumin (HSA), although it should be noted that other phenolic acids demonstrated similar or higher binding above that observed for ellagic acid (Tang et al., 2013, Tang et al., 2016, Peng et al., 2016). The binding constant of EGCG to bind to HSA was calculated independently at both above (11.0 10^4 L/Mol) and below (5.81 10^4 L/Mol) the value reported for ellagic acid (Ozyurt et al., 2016, Zinellu et al., 2014). It is also worth noting that nearly all polyphenols exhibit a higher binding affinity, as

measured by binding constant, for human serum albumin over bovine serum albumin. This being said in all cases the polyphenol had a higher affinity for serum of any kind than for haemoglobin (Ozyurt et al., 2016, Zinellu et al., 2014). This suggests that there is specificity with regards to the type of biological protein that polyphenols will interact with. Furthermore, modification of the serum albumin, such as more glycated HSA in diabetes patients, has been associated with the reduced binding affinity for polyphenols (Xu et al., 2014b, Xie et al., 2012), with EGCG being among those shown to exhibit this effect (Singha Roy et al., 2016). Overall, there is accumulating data that demonstrates polyphenols can bind to serum proteins and that specific structural features such as methylation or galloylation increase this binding. A summary of polyphenol-serum protein interactions can be seen in Table 4.1.

Table 4.1: Summary of polyphenol interactions with plasma proteins

Polyphenol name	Binding partner	Binding constant (10^4 L/Mol)	Ref.
4-caffeoylquinic acid	Human serum albumin	24.7	(Tang et al., 2016)
3-caffeoylquinic acid	Human serum albumin	19.7	(Tang et al., 2016)
5-caffeoylquinic acid	Human serum albumin	10.3	(Tang et al., 2016)
Resveratrol	Human serum albumin	74.3	(Lu et al., 2007)
	Bovine serum albumin	2.5	(Bourassa et al., 2010)
	Haemoglobin	7.4	(Lu et al., 2007)
Rosmarinic acid	Human serum albumin	4.29	(Peng et al., 2016)
Ellagitannin (dp1)	Bovine serum albumin	1.84	(Karonen et al., 2015)
Ellagitannin (dp2)	Bovine serum albumin	0.57	(Karonen et al., 2015)
Ellagitannin (dp3)	Bovine serum albumin	0.76	(Karonen et al., 2015)
Ellagitannin (dp5)	Bovine serum albumin	2.32	(Karonen et al., 2015)
Ellagitannin (dp6)	Bovine serum albumin	1.69	(Karonen et al., 2015)
Ellagitannin (dp8)	Bovine serum albumin	1.69	(Karonen et al., 2015)
Procyanidin dp2	Bovine serum albumin	0.39	(Watrelot et al., 2015)
Procyanidin dp8	Bovine serum albumin	1.70	(Watrelot et al., 2015)

Polyphenol name	Binding partner	Binding constant (10^4 L/Mol)	Ref.
EC	Human serum albumin	1.40	(Ozyurt et al., 2016)
		1.64	(Zinellu et al., 2014)
	Human Haemoglobin	0.37	(Ozyurt et al., 2016)
	Bovine serum albumin	0.046	(Watrelot et al., 2015)
		0.47	(Zinellu et al., 2014)
EGC	Human serum albumin	0.60	(Ozyurt et al., 2016)
		2.15	(Zinellu et al., 2014)
	Human Haemoglobin	0.24	(Ozyurt et al., 2016)
	Bovine serum albumin	0.64	(Zinellu et al., 2014)
ECG	Human serum albumin	10.3	(Zinellu et al., 2014)
	Bovine serum albumin	3.37	(Zinellu et al., 2014)
EGCG	Human serum albumin	5.81	(Ozyurt et al., 2016)
		11.0	(Zinellu et al., 2014)
	Bovine serum albumin	3.68	(Zinellu et al., 2014)
	Human Haemoglobin	1.53	(Ozyurt et al., 2016)
Vescalagin	Bovine serum albumin	5.2	(Dobreva et al., 2014)
Pedunculagin	Bovine serum albumin	4.2	(Dobreva et al., 2014)
Ellagic acid	Human serum albumin	8.47 ± 0.52	(Tang et al., 2013)
Genistein	Bovine serum albumin	1.26(+/-0.3	(Bourassa et al., 2010)
Curcumarin	Bovine serum albumin	3.33(+/-0.8)	(Bourassa et al., 2010)

4.2.4 Implications of polyphenol interactions with proteins

The literature has suggested that plasma protein may act as transporters which bind to polyphenols increasing the polyphenol concentration in plasma (Lu et al., 2007). For example, quercetin was reported to bind to haemoglobin after passive diffusion into blood cells, with the blood cells acting as reservoirs (Fiorani et al., 2003). Numerous reports discuss the impact of polyphenol-protein interactions on the bioactivity and bioavailability of the polyphenols (Xiao et al., 2011). Some reports suggest the concentration of tea or cocoa polyphenols in the plasma is unaltered by the addition of milk prior to intake (Keogh et al., 2007, Kyle et al., 2007). Others report that the antioxidant capacity of the plasma was found to decrease, although this was not replicable in vitro (Serafini et al., 1996) and not all

reports saw this effect (Reddy et al., 2005). However, it is important to consider that this observation appears very method dependent (Bourassa et al., 2013).

There are reports of polyphenols binding to biologically relevant proteins, altering the protein function. The green tea catechins were reported to directly bind and modify the protein disulfide isomerase isoformA3 (PDIA3), causing moderate inhibition of the protein activity. In this study once again the affinity for the protein was higher for galloylated catechins compared to non-galloylated catechins (Trnkova et al., 2013). The enzymatic action of porcine pancreatic elastase was inhibited by binding with procyanidin, where an increase in the degree of polymerisation was associated with an increase in protein action inhibition. (Bras et al., 2010). In some cases polyphenols can alter protein activity after binding by modifying the protein structure as was the case with the binding of curcumin to protein kinase C. The modification was associated with increases in the protein activity (Majhi et al., 2010).

One study reports the effects of serum proteins on a polyphenol-ligand interaction. A quinoline appended chalcone derivative was found to exhibit strong inhibition of angiotensin converting enzyme (ACE) activity however this inhibition was diminished in the presence of BSA (Kumar et al., 2015). At the time of writing this was the only research article reporting the influence of serum proteins on the interaction of a polyphenol with a biological protein. However, non-protein compounds have been found to alter polyphenol-protein interactions. For example, Goncalves et al. found that the presence of pectin caused the dissociation of previously bound procyanidin-trypsin complex (Goncalves et al., 2011). Although limited, the data suggests that polyphenol binding to a ligand can be interrupted by external compounds, including serum proteins.

4.2.5 Objectives

This chapter aims to determine whether 1) EGCG is able to inhibit VEGF-induced angiogenesis under the serum protein rich conditions required for *ex vivo* aortic ring growth; and 2) the presence of FCS can affect the strong inhibition of VEGF-stimulated VEGFR-2 phosphorylation demonstrated by EGCG and EA in serum-free media as reported in chapter 2.

4.3 Material and methods

4.3.1 Determining effect of EGCG on *ex vivo* angiogenesis in the aortic ring assay

The aortic ring assay was performed as previously reported (Baker et al., 2012).Per repeat 3 whole aortas were removed from 8 week old C57BL/6J mice following death by rising CO_2 and transferred straight to chilled Opti-MEM media (Thermo Fisher Scientific, UK) for transport. The aortas were cleaned and cut into 5 mm wide rings using microscopy while submerged in Opti-MEM media under sterile conditions. The rings were transferred to fresh, sterile Opti-MEM media in a 10 cm dish and incubated at 37 °C, 5 % CO_2 for 24 hours. The next day 50 µl DMEM containing 0.1 mg/ml rat tail collagen was added to each well of a 96 well plate and one ring imbedded into each well and the plate was incubated at 37 °C, 5 % CO_2 for 1 hour to set. After the collagen was set each well was fed with 150 µl of the treatment solution containing 2.5 % FCS and either no VEGF for a negative control ('VEGF(-)'), VEGF at 30 ng/ml concentration for a VEGF control ('VEGF (+)'), VEGF at 30 ng/ml and the EGCG as a treatment ('VEGF + EGCG') or VEGF at 30 ng/ml and epicatechin (EC) as a negative polyphenol control, considering EC was previously unable to inhibit VEGF induced VEGFR-2 phosphorylation (Cerezo et al., 2015) ('VEGF + EC'). Where polyphenol was included with the treatment it was allowed to mix in the solution for 5 minutes at room temperature before being added to the 96 well plate. The media was replaced with freshly prepared media on day 3 and day 6. On day 7 the wells were washed with PBS+$CaCl_2$+$MgCl_2$ and fixed with 4 % formalin for 30 minutes. The wells were blocked and the endothelial cells were stained with 0.1 mg/ml BS1 lectin conjugated to FITC for 24 hours at 4°C. Imaging was performed in situ using the AxioVision software on an inverted fluorescence microscope (Zeiss) and quantification of microvessels undertaken in a blinded manner. A visual representation of the protocol including examples of tissue preparation, bright field images of growing tissue, and fluorescent imaging of stained tissue used for quantification can be seen in Figure 4.1.

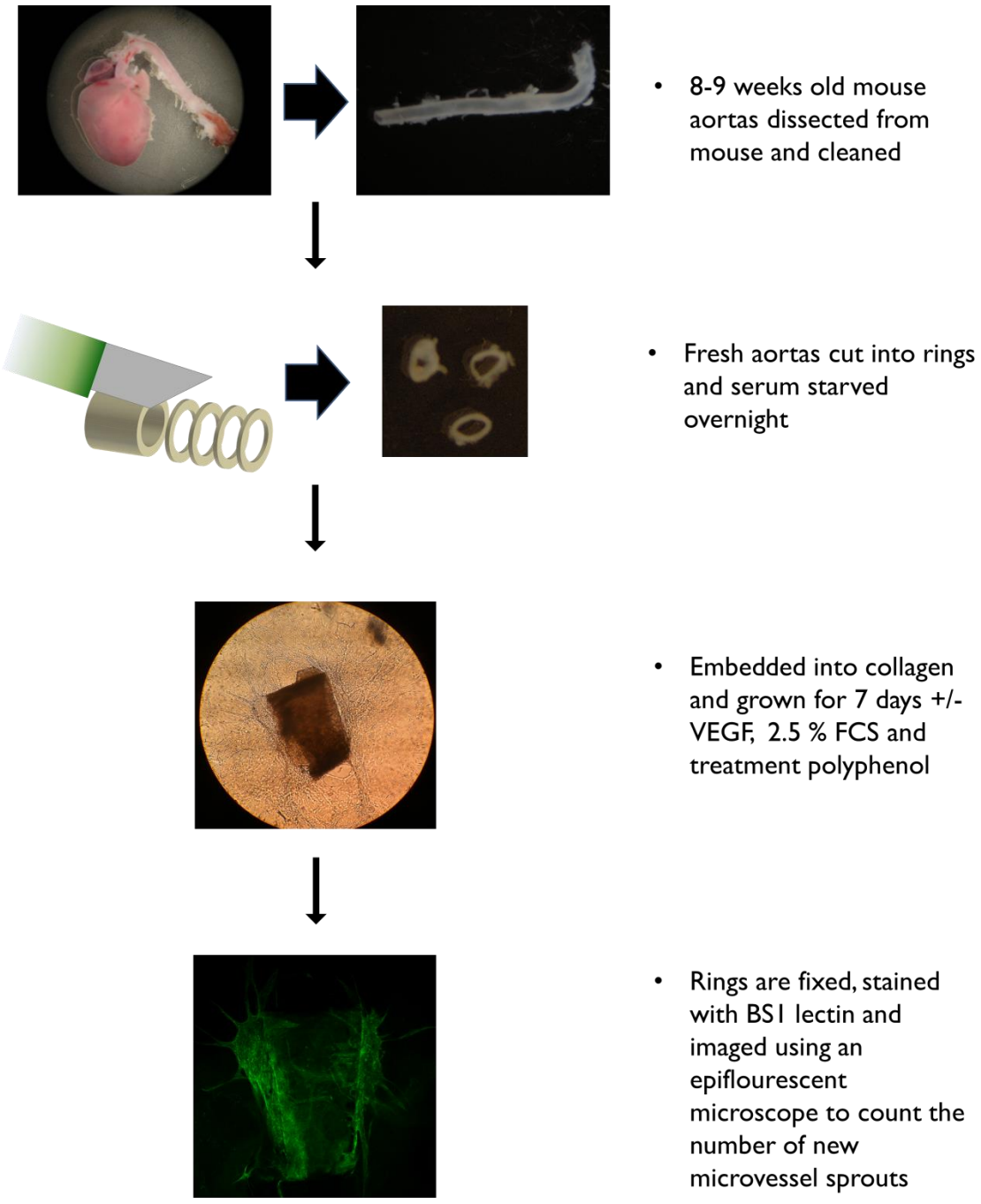

Figure 4.1: A visual representation of the experimental procedure described in section 4.3.1.

4.3.2 Subculture of HUVECs

HUVECs were subcultured as described earlier in section 2.3.1 in either T75 or T175 and always seeded to a density of 3500 cells per $cm^{2.}$

4.3.3 Culture of cells in 6 well plates for treatment

HUVECs at passage 5 were grown to 100 % confluency in 6 well plates as described previously in section 2.3.2 and always seeded to a density of 3500 cells per cm^2.

4.3.4 Determining the effect of serums proteins of polyphenol inhibition of VEGF signalling

For every well requiring treatment 2.5 ml of basal media was transferred to sterile flasks labelled with the treatment and these were warmed in a water bath. A fresh aliquot of VEGF was defrosted on ice and added to the relevant treatments at a concentration of 1:2000 to give a final VEGF concentration of 25 ng/ml in the VEGF positive treatments. Foetal calf serum was added to the treatments requiring FCS prior to polyphenol addition at a 2 % concentration (called 'VEGF/FCS + PP') after which the 1000 x concentrated polyphenol stocks were added to the appropriate treatments at a 1:1000 ratio and allowed to mix with the VEGF for 5 minutes at room temperature. DMSO was added as a vehicle control to the VEGF negative and VEGF positive controls. One treatment (called 'VEGF/PP + FCS') allowed the VEGF and polyphenol to mix for 5 minutes before the addition of FCS. Confluent HUVECs grown in 6 well plates (Greiner BioOne Ltd.) were washed twice with 2 ml PBS warmed to 37 °C. After the 5 minute pre-mixing of the VEGF and polyphenol FCS was added at 2 % to the 'VEGF/PP + FCS' treatment and all treatments were added to the appropriate wells and incubated at 37 °C, 5% CO_2 for 5 minutes. 2 plates were used per experiment and the whole experiment was repeated at least 3 times. For a visual representation of the experimental protocol please see Figure 4.2.

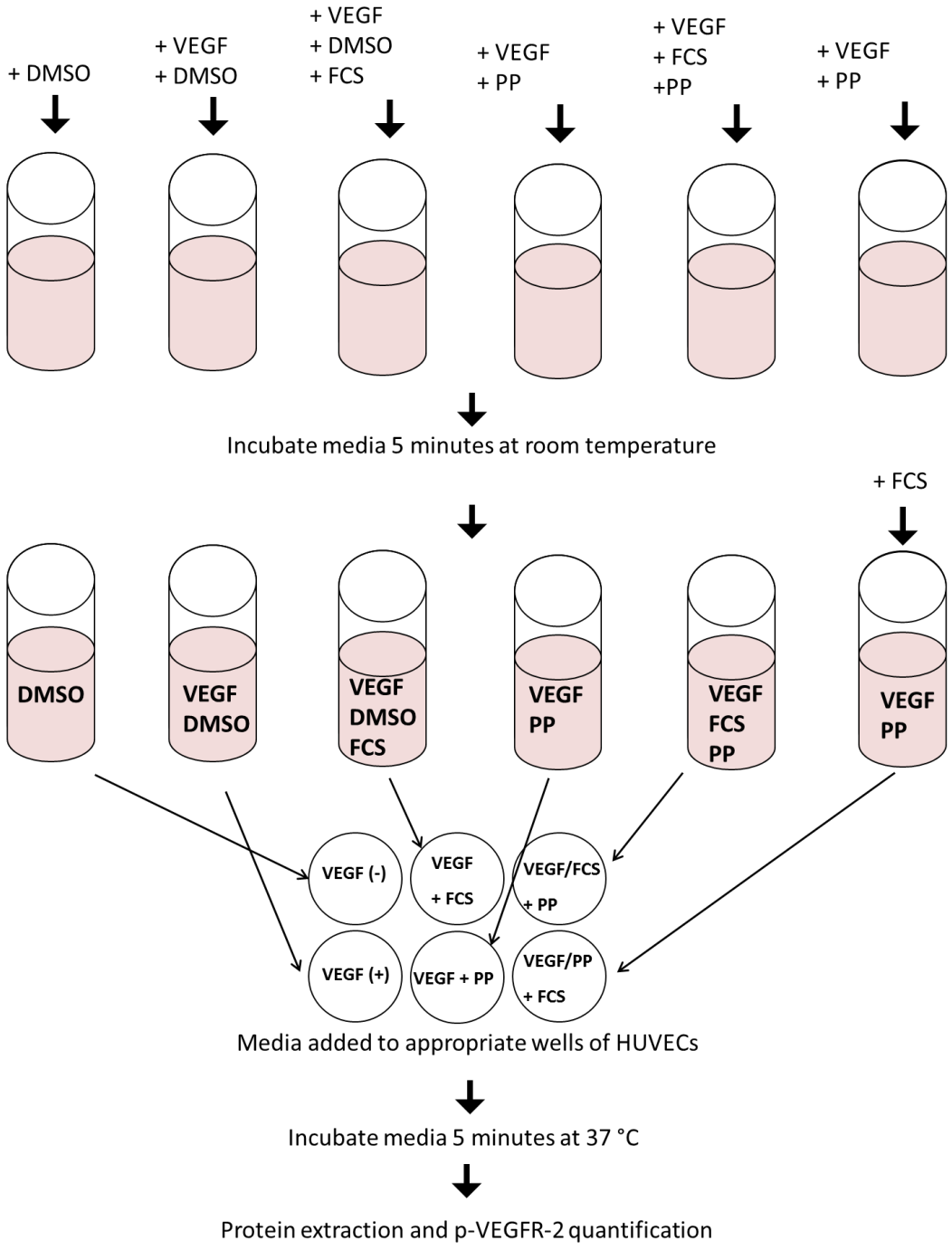

Figure 4.2: A visual representation of the experimental procedure described in section 4.3.4.

4.3.5 Determining the polyphenol inhibition of VEGF signalling following longer incubation of VEGF/PP complex with FCS

2 x 20 ml and 1 x 10 ml basal media was transferred to 3 sterile flasks. The 20 ml volumes were labelled VEGF/FCS + PP and VEGF/PP + FCS while the 10 ml volume was labelled VEGF only. The VEGF was defrosted on ice and added to the relevant treatments at a concentration of 1:2000 to give a final VEGF concentration of 25 ng/ml. FCS was added at a 2 % concentration to the appropriate media (VEGF/FCS + PP treatment) prior to the addition of the polyphenol. EGCG and EA stocks were added to the appropriate treatments at a 1µM concentration and allowed to mix for 5 minutes at room temperature. One treatment (called 'VEGF/PP + FCS') allowed the VEGF and polyphenols to mix for 5 minutes before the addition of FCS. DMSO was added as a vehicle control to the VEGF negative and VEGF positive controls. After this 5 minutes and immediately prior to cell treatment FCS was added to the 'VEGF/PP + FCS' treatment at a 2 % concentration. Treatments were added to confluent HUVECs grown in 6 well plates (Greiner BioOne Ltd.) as shown in Figure 4.3 and incubated at 37 °C, 5% CO_2 for 5 minutes. The VEGF/FCS + PP and VEGF/PP + FCS, VEGF only solutions were then incubated between treatments at 37 ˚C and 5 % CO_2 and the treatment were repeated using the incubated media at 1 h, 2 h and 4 h post polyphenol addition. For a visual representation of the experimental protocol please see Figure 4.3.

4.3.6 Cell lysis

The cells were washed with 2 ml ice cold PBS twice after which 50 µl cell lysis buffer supplemented with 1 mM PMSF according to manufacturer's instructions (#9803, Cell Signalling Technology, Inc). Plates were scraped (541070, Greiner BioOne Ltd.) and the lysates collected and kept on ice for processing. Each lysate was vortexed for 20 seconds, three times at maximum speed with breaks on ice between each vortex. The lysates were then centrifuged for 10 minutes at 13,000 x g at 4 °C and the supernatant was collected in a separate tube. A 5 µl aliquot of each sample was taken prior to freezing to use to determine the total protein concentration in each sample. The sample and 5 µl aliquot were frozen and stored at -80 °C until analysis could be performed.

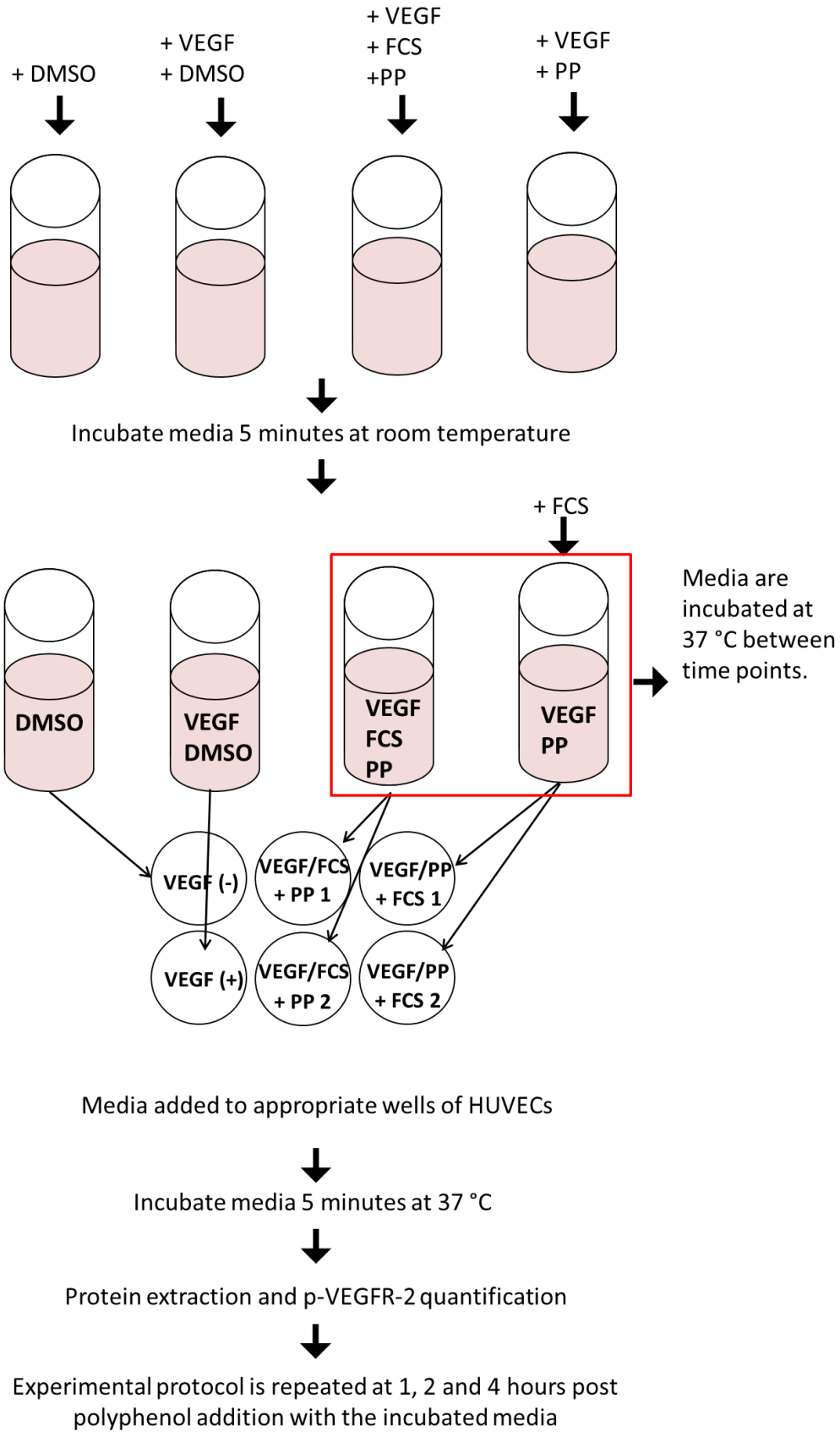

Figure 4.3: A visual representation of the experimental procedure described in section 4.3.5.

4.3.7 Quantification of the absolute protein concentration of cell lysates

To quantify the total protein a sample the bicinchoninic acid (BCA) assay was used as described previously in section 2.3.5. Briefly, a BSA calibration curve ranging from 0 mg/ml to 1 mg/ml was set up and each experiemtn 5 µl sample was diluted in 55 µl of NaPi buffer. The reaction solution of Bicinchoninic acid and copper II sulphate in a ratio of 50:1 v:v was added to 25 µl of each sample in duplicate and the reaction was incubated at 37 °C for 30 minutes after which it was immediately read using a FluorStar OPTIMA spectrophotometer (BMG Labtech) at 450nm.

4.3.8 Determining the amount of VEGF receptor phosphorylation in HUVECs following cell treatments

An enzyme linked immunosorbent assay (ELISA) kit was used (7335, New England Biolabs) to determine the amount of tyrosine 1175 (Y1175) VEGFR-2 phosphorylation in the cell lysates was used as previously described in section 2.3.6. Each experimental sample was performed in duplicate and following completion of the ELISA protocol the plates were read immediately at 450nm-540 nm on a FluorStar OPTIMA spectrophotometer (BMG Labtech). The percentage VEGFR-2 phosphorylation in comparison to a VEGF stimulated sample was calculated.

4.3.9 Statistical analysis

All samples were compared to the VEGF only stimulated samples and presented as a percentage of this value. To determine statistical significance of any differences ANOVA with a Tukey post-hoc test was conducted. Results were considered significant with a p value of under 0.05.

4.4 Results

4.4.1 EGCG was unable to inhibit VEGF-induced new vessel growth in aortic ring assay

The effect of EGCG treatment in the aortic ring assay was assessed. Epicatechin, a polyphenol previously reported as incapable of inhibiting VEGF-induced VEGFR-2 phosphorylation (Cerezo et al., 2015), was used as a polyphenol control. Treatment with 30 ng/ml VEGF + FCS significantly induced more new vessel growth that FCS only treatment. Treatment with EGCG + FCS also induced a smaller but significant increase in microvessel number. Treatment with VEGF + EGCG and VEGF + EC induced a strongly significant increase in number of microvessel in comparison the FCS only treatment but did not differ from the VEGF + FCS treatment. EGCG and epicatechin, and both the polyphenol treatments were not significantly different to the VEGF negative or VEGF positive controls. The variation in the sample was high, as is expected with this assay (see Figure 4.4).

4.4.2 Potent inhibition of VEGF signalling by epigallocatechin gallate does not occur in the presence of FCS

Considering that the addition of FCS to the media as required for aortic ring growth may have contributed to the lack of observed inhibition of VEGF-induced angiogenesis by EGCG, the effect of FCS in the simpler HUVEC model of VEGF-induced VEGFR-2 phosphorylation was investigated. Addition of FCS to a VEGF only treatment ('VEGF/FCS') did not significantly increase the VEGFR-2 phosphorylation but did increase the variability. To determine the effect of FCS on this inhibition FCS was added to the media either before the pre-mixing of the VEGF and EGCG or after this 5 minute period. Where FCS was added to the media prior to the mixing of the VEGF and EGCG ('VEGF/FCS + EGCG') the VEGF was able to stimulate VEGFR-2 phosphorylation at a comparable amount to a VEGF only treatment. However when the VEGF and EGCG were allowed to mix for 5 minutes prior to the addition of the FCS ('VEGF/PP + FCS') a comparative inhibition to the VEGF + EGCG treatment was achieved after 5 minute treatment (Figure 4.5).

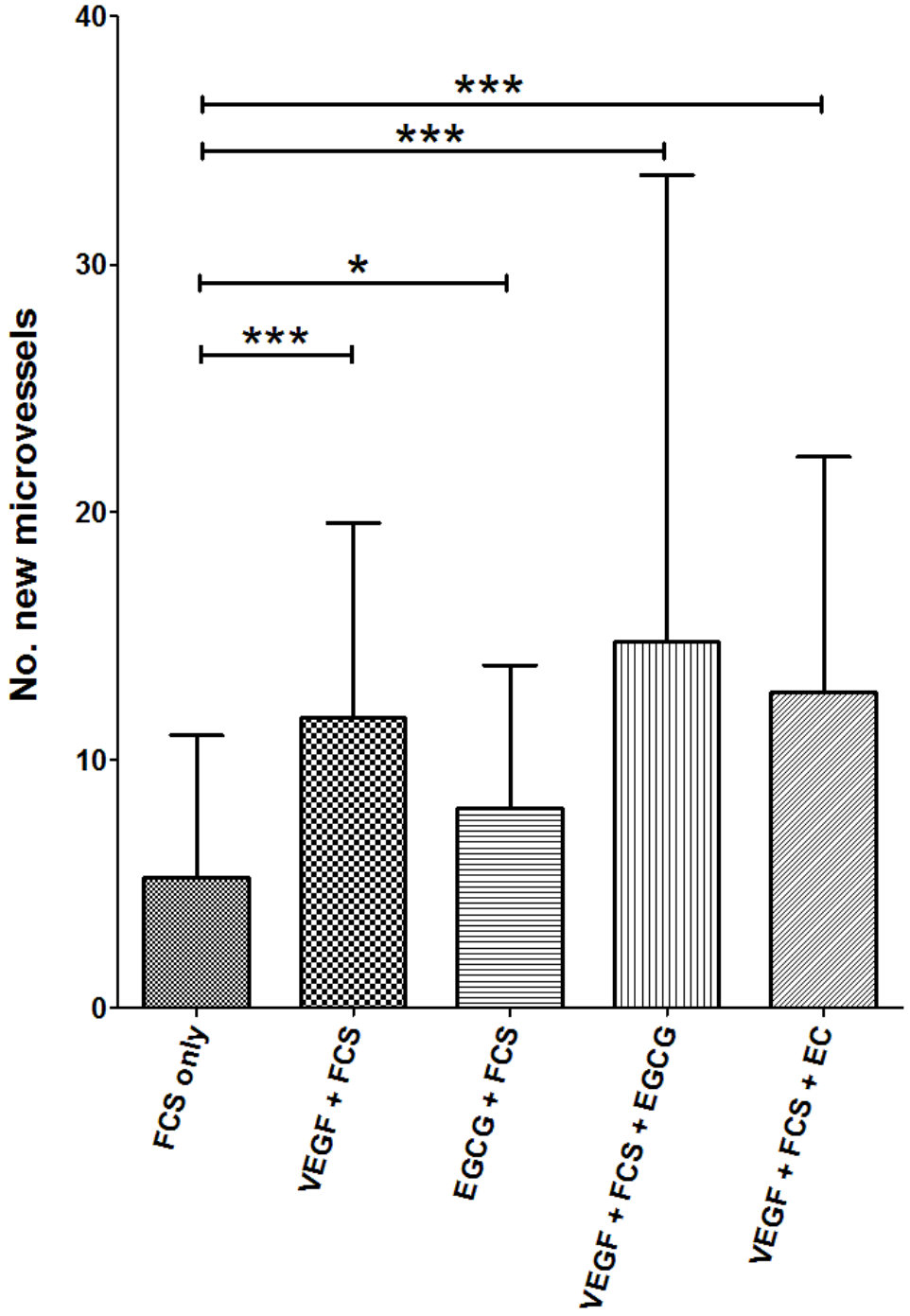

Figure 4.4: Effect of EGCG and epicatechin (EC), on VEGF induced new microvessel growth in the aortic ring assay

5 mm thick mouse aortic rings from 8 week old C57BL/6J mice were embedded into collagen and grown in 2.5% supplemented Opti-MEM media in the presence or absence of VEGF (30 ng/ml) and/or polyphenols (1 µM) for 7 days. The number of new microvessels after this time and a Kruskal-Wallis ANOVA with Dunns post-test was undertaken. Data is presented as mean ± SD. The results were plotted into a bar graph * = $p < 0.05$; n = 50 - 76.

4.4.3 Potent inhibition of VEGF signalling by ellagic acid does not occur in the presence of FCS

As in section 4.4.1 the addition of FCS to a VEGF only ('VEGF/FCS') treatment did not significantly increase the VEGFR-2 phosphorylation but did increase the variability. However, when FCS was present in the media prior to the addition of ellagic acid ('VEGF/FCS + EA') the VEGF was able to stimulate VEGFR-2 phosphorylation to the same amount as with the VEGF only treatment. Interestingly, the 'VEGF/EA + FCS' treatment, where ellagic acid was pre-mixed with the VEGF for 5 minutes prior to the addition of FCS to the media, was significantly different to both the 'VEGF/FCS +EA' ($p = \leq 0.001$) and the 'VEGF/EA' ($p = \leq 0.05$) treatments. The VEGF in the 'VEGF/EA + FCS' treatment was able to stimulate around 28 % of the VEGFR-2 phosphorylation observed in the VEGF only treated cells (Figure 4.6).

4.4.4 Combinations of EGCG and EA treatment does not improve VEGF inhibition capacity of the polyphenols in presence of FCS

The effect of FCS on the inhibition of VEGF-induced VEGFR-2 phosphorylation by a combination of 1µM EGCG and ellagic acid was also investigated. As seen before FCS addition to a VEGF only ('VEGF/FCS') treatment did not significantly increase the VEGFR-2 phosphorylation but did increase the variability. When 2 % FCS was added to the VEGF supplemented media prior to the addition of the polyphenols ('VEGF/FCS + EA/EGCG') there was no significant difference in the VEGFR-2 phosphorylation in comparison to the VEGF only treatments. As seen with EGCG only treatment (section 4.4.1; Figure 4.5), when the VEGF and polyphenols were pre-mixed for 5 minutes before to the addition of FCS to the treatment ('VEGF/EA/EGCG + FCS') the VEGFR-2 phosphorylation was completely inhibited with no significant difference to the VEGF/EA/EGCG treatment arm (Figure 4.7).

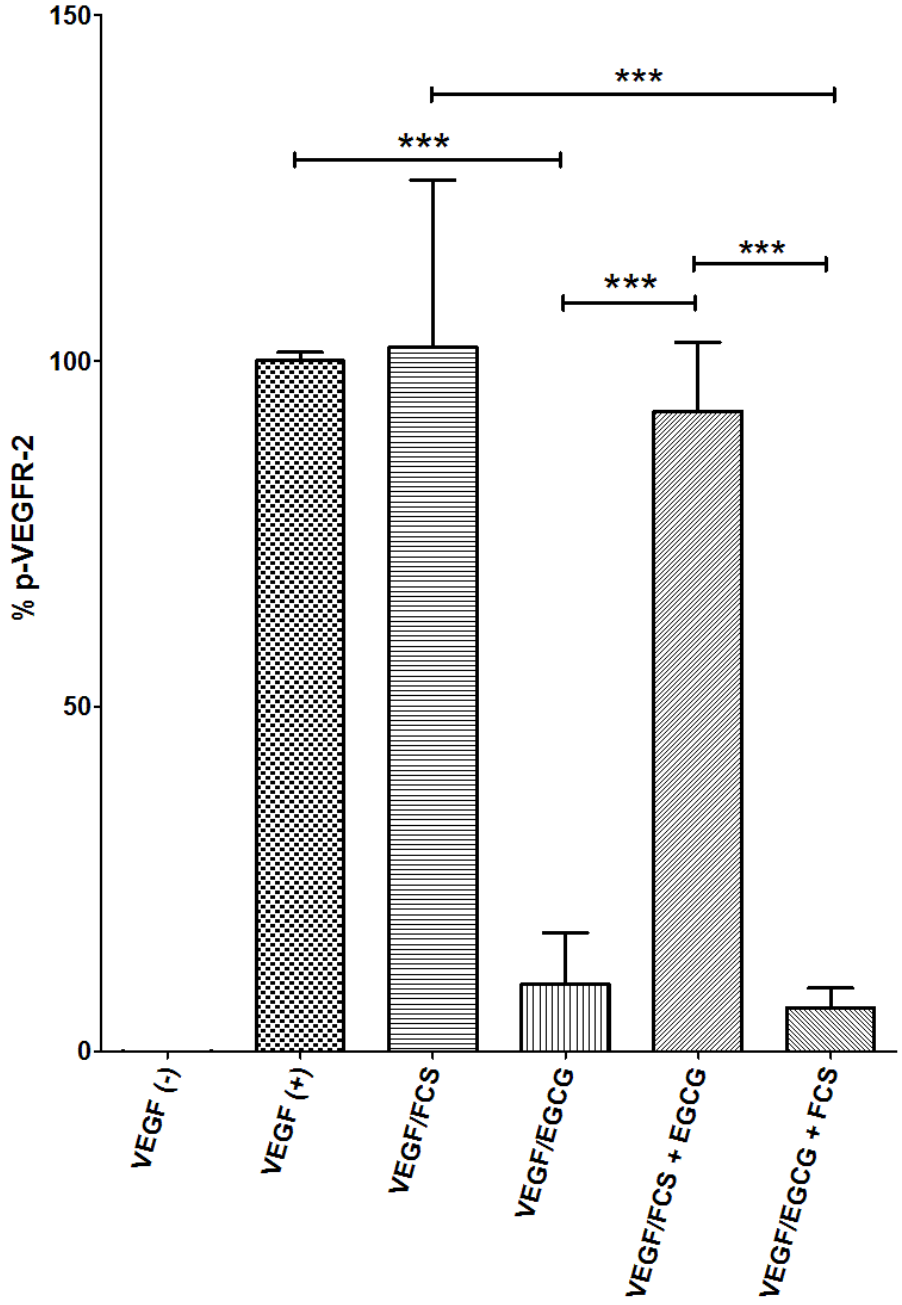

Figure 4.5: Effect of FCS on EGCG inhibition of VEGF induced VEGFR-2 phosphorylation

25 ng/ml VEGF supplemented EBM-2 media (Lonza) was supplemented further with 2% FCS as appropriate. EGCG was then added to the appropriate solutions for 5 minutes to mix, after which one treatment with VEGF and EGCG only, ' VEGF/EGCG + FCS' had FCS added to 2 % concentration. HUVECs were stimulated for 5 minutes at 37 °C, 5 % CO_2. Cells were then lysed and total protein collected. VEGFR-2 phosphorylation was quantified in samples normalised to the same total protein by sandwich ELISA and a percentage phosphorylation in comparison to the VEGF only stimulated sample was calculated. These were plotted into a bar graph and a one-way ANOVA with Tukey post-hoc statistical test performed. *** = $p \geq 0.001$; n = 4.

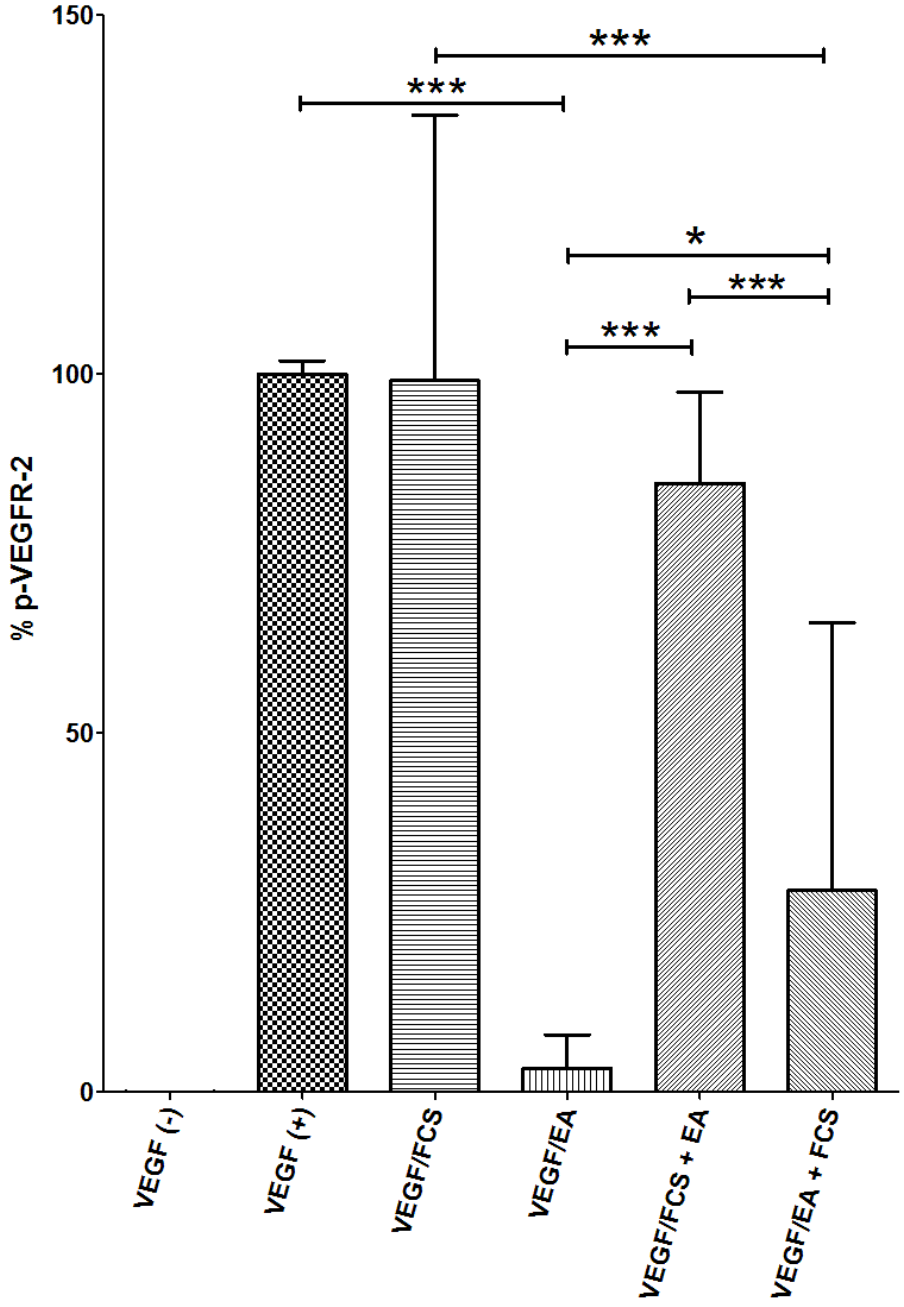

Figure 4.6: Effect of FCS on EA inhibition of VEGF induced VEGFR-2 phosphorylation

25 ng/ml VEGF supplemented EBM-2 media (Lonza) was supplemented further with 2% FCS as appropriate. EA was then added to the appropriate solutions for 5 minutes to mix, after which one treatment with VEGF and EA only ' VEGF/EA + FCS' had FCS added to 2 % concentration. HUVECs were stimulated for 5 minutes at 37 °C, 5 % CO_2. Cells were then lysed and total protein collected. VEGFR-2 phosphorylation was quantified in samples normalised to the same total protein by sandwich ELISA and a percentage phosphorylation in comparison to the VEGF only stimulated sample was calculated. These were plotted into a bar graph and a one-way ANOVA with Tukey post-hoc statistical test performed. *** = $p \leq 0.001$; * = $p \leq 0.05$; n = 8.

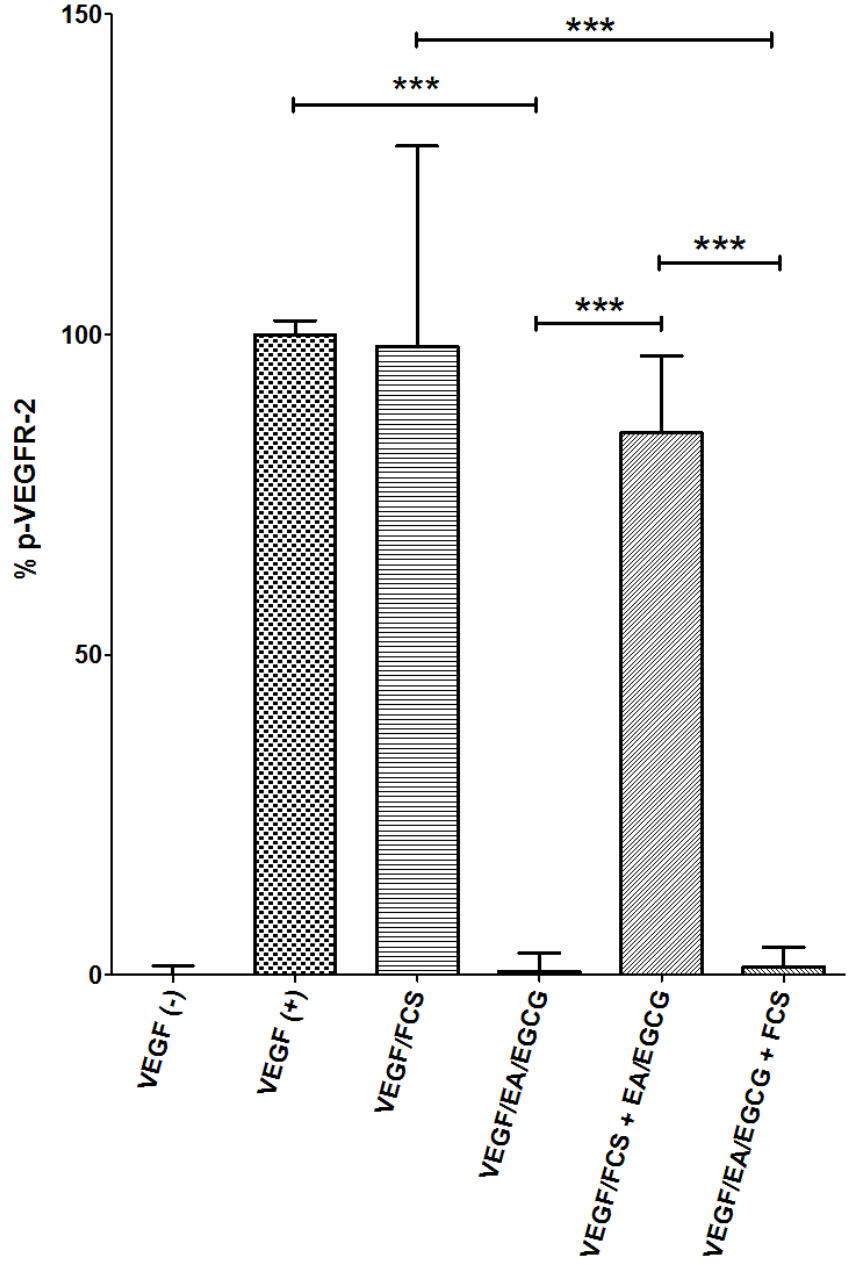

Figure 4.7: Effect of FCS on EA and EGCG combined inhibition of VEGF induced VEGFR-2 phosphorylation

25 ng/ml VEGF supplemented EBM-2 media (Lonza) was supplemented further with 2% FCS as appropriate. EA and EGCG were then added to the appropriate solutions for 5 minutes to mix, after which one treatment with VEGF, EA, and EGCG only ' VEGF/EA/EGCG + FCS' had FCS added to 2 % concentration. HUVECs were stimulated for 5 minutes at 37 °C, 5 % CO_2. Cells were then lysed and total protein collected. VEGFR-2 phosphorylation was quantified in samples normalised to the same total protein by sandwich ELISA and a percentage phosphorylation in comparison to the VEGF only stimulated sample was calculated. These were plotted into a bar graph and a one-way ANOVA with Tukey post-hoc statistical test performed. *** = $p \leq 0.001$; * = $p \leq 0.05$; n = 6.

4.4.5 VEGF regains VEGFR-2 phosphorylating activity over time in the presence of FCS following complete inhibition by EGCG/EA

To determine the effect of FCS on EGCG and ellagic acid combined inhibition of VEGF-induced VEGFR-2 phosphorylation over time the treatments were repeated as in section 4.4.3, stored at 37 °C and 5 % CO_2, and at regular time intervals were removed to perform a 5 minute treatment on HUVECs. As observed previously after 5 minutes the treatments where EGCG and ellagic acid were mixed with the VEGF before the addition of FCS completely inhibited VEGFR-2 phosphorylation while the treatments where the polyphenols were mixed with the VEGF/FCS solution were unable to inhibit VEGFR-2 phosphorylation, in fact a small but significant increase in p-VEGFR-2 was observed in the 'VEGF/FCS + EGCG/EA' treatment at 5 minutes. Over time it was observed that the VEGF in the 'VEGF/EGCG/EA + FCS' treatment, which was completely inhibited at the 5 minute time point, regained activity. This increase in VEGF-stimulated VEGFR-2 phosphorylation was only significant however after 4 hours ($p = \leq 0.05$). The VEGFR-2 phosphorylation of the 'VEGF/FCS + EGCG/EA' treatment after 1, 2 and 4 hours incubation was significantly lower than the same treatment at 5 minutes, although only after 4 hours was this 'VEGF/FCS + EGCG/EA' treatment significantly different to the VEGF only 'VEGF (+)' treatment (Figure 4.8).

4.4.6 Sustained VEGF activity in the presence of FCS, EGCG and EA *in vitro*

The VEGFR-2 phosphorylation stimulated by the 'VEGF/FCS + EGCG/EA' treatment after 1 h, 2 h, and 4 h incubation time was significantly higher than the stimulation by the 'VEGF (+)' treatment incubated for the same duration. Treatment of cells with the 'VEGF (+)' media after 4 hours incubation at 37 °C, 5 % CO_2 only induced a small amount of VEGFR-2 phosphorylation but this was not significantly different to the 'VEGF (-)' treatment. The 'VEGF/FCS + EGCG/EA' treatment retained over 75 % VEGFR-2 stimulation activity after the same 4 hour incubation. However, there was a smaller but significant decline in VEGF activity over time in the 'VEGF/FCS + EGCG/EA' treatment with the VEGF-induced VEGFR-2 phosphorylation of this treatment being significantly different to the 'VEGF (+)' treatment after 4 h incubation at 37 °C, 5 % CO_2. (Figure 4.9).

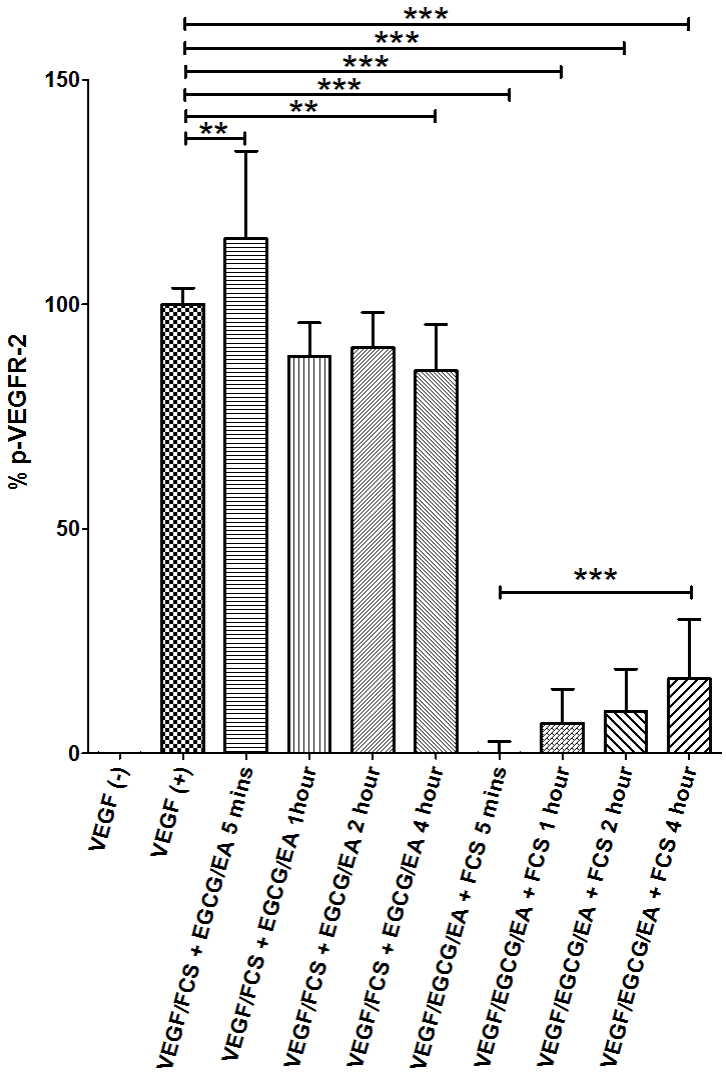

Figure 4.8: Effect of FCS on EA and EGCG combined inhibition of VEGF induced VEGFR-2 phosphorylation over time

A negative control lacking VEGF ('VEGF (-)') and a 25 ng/ml VEGF only control ('VEGF (+)') were set up. To create the 'VEGF/FCS + EGCG/EA' treatment 25 ng/ml VEGF supplemented EBM-2 media was supplemented further with 2% FCS, after which 1 µM EA and EGCG were then added for 5 minutes to mix. To create the 'VEGF/EGCG/EA + FCS' treatment 25 ng/ml VEGF supplemented EBM-2 media was supplemented with 1 µM EGCG and EA and allowed to mix for 5 minutes, after it was further supplemented with 2% FCS. HUVECs were stimulated with all treatments for 5 minutes at 37 °C, 5 % CO_2 and the remaining media were left to incubate at 37 °C, 5 % CO_2. Periodically at 1, 2, and 4 hours after the addition of the polyphenol the media were used to treat HUVEC for 5 minutes at 37 °C, 5 % CO_2. After each treatment cells were then lysed and total protein collected. VEGFR-2 phosphorylation was quantified in samples normalised to the same total protein by sandwich ELISA and a percentage phosphorylation in comparison to the VEGF only stimulated sample was calculated. These were plotted into a bar graph and a one-way ANOVA with Tukey post-hoc statistical test performed. *** = $p \leq 0.001$; ** = $p \leq 0.01$; n = 6.

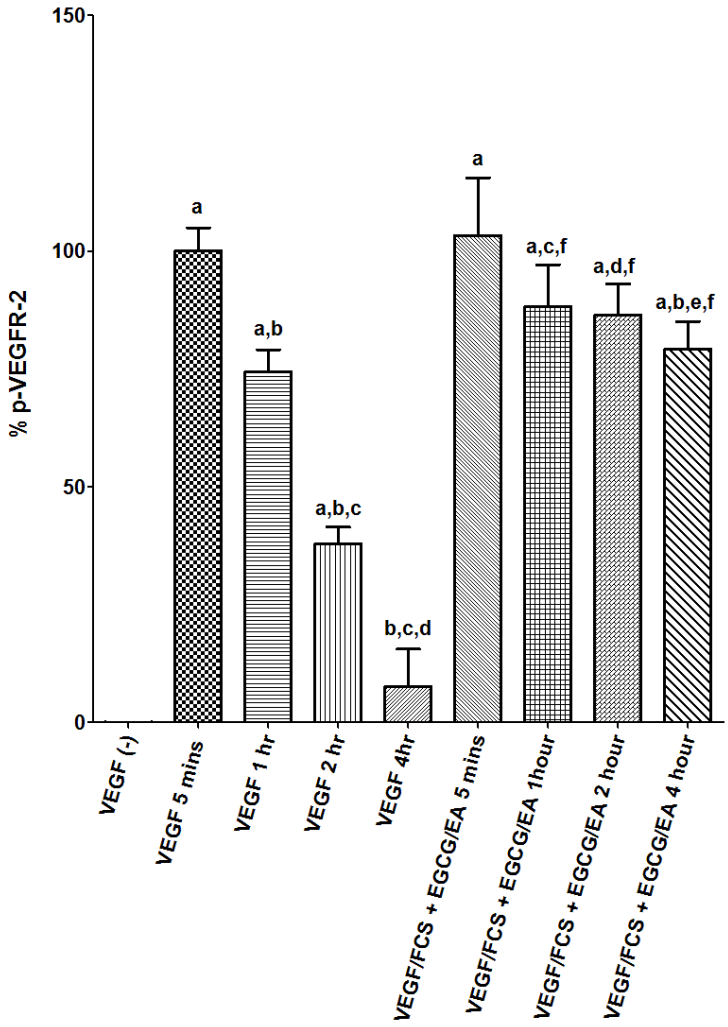

Figure 4.9: The loss of VEGFR-2 phosphorylation activity of VEGF over time in cell culture media

25 ng/ml VEGF supplemented EBM-2 media (Lonza) was supplemented further with 2% FCS as appropriate and EA and EGCG were then added to the FCS containing treatments. The treatment solutions were incubated at 37 °C, 5 % CO2 until needed. A VEGF only treatment sample was incubated for the same duration as a comparison. HUVECs were stimulated for 5 minutes at 37 °C, 5 % CO2 at 5 minutes, and 1, 2, and 4 hours incubation. After each treatment cells were lysed and total protein collected. VEGFR-2 phosphorylation was quantified in samples normalised to the same total protein by sandwich ELISA and a percentage phosphorylation in comparison to the VEGF only stimulated sample was calculated. These were plotted into a bar graph and a one-way ANOVA with Tukey post-hoc statistical test performed. a represent a significant difference to the 'VEGF (-)' treatment; b represents a significant difference to the 'VEGF' 5 min treatment; c represents a significantly different to the 'VEGF' 1 hr treatment; d represents a significant difference to the 'VEGF' 2hr treatment; e represents a significant difference to the 'VEGF' 4 hr treatment; f represents a significant difference to the 'VEGF/FCS + EGCG/EA' 5 min treatment; n = 6.

4.5 Discussion

In chapter 2 it was identified that both EGCG and ellagic acid were able to potently inhibit VEGF-induced VEGFR-2 phosphorylation in HUVECs primarily through interactions between the VEGF and the polyphenol. Please see Figure 5.1 for a diagrammatic representation of the proposed mechanism of action for the inhibition of VEGF-induced VEGFR-2 phosphorylation by polyphenols in serum free media. Considering the critical role for angiogenesis in atherosclerosis chapter 3 investigated the inhibitory effects of the polyphenols on VEGF activity in relevance to the progression of atherosclerosis. Although injection of VEGF did not induce atherosclerosis in the model it is widely reported that upregulation of endogenous VEGF signalling occurs within the atherosclerotic plaque. However, no inhibition of atherosclerosis progression was observed in any treatment arm through dietary intervention with EGCG and ellagic acid, and only a minimal effect was observed when a high dose of EGCG and EA was delivered by intraperitoneal injection. The aim of this chapter was to provide an explanation for the lack of observed inhibition of the anticipated endogenous VEGF upregulation in atherosclerosis. Firstly, it was investigated whether angiogenesis could be inhibited by the potent VEGF inhibitor EGCG and, following the lack of inhibitory effect observed in the aortic ring assay, whether the interaction of the polyphenols with serum proteins could alter the polyphenol inhibition of VEGF-induced VEGFR-2 phosphorylation.

4.5.1 Polyphenol inhibition in the aortic ring assay

Through the use of the aortic ring assay it was established that EGCG was not able to inhibit angiogenesis and reduce new vessel growth in comparison to the VEGF stimulated treatment. EGCG treatment was also not able to significantly alter the new vessel generation in comparison to treatment with epicatechin, a polyphenol unable to inhibit VEGF induced VEGFR-2 phosphorylation *in vitro*. The main difference between the treatment media used in the HUVEC experiments and that used in the aortic ring assay was the presence of 2.5 % FCS as necessary for the survival of the aortic ring tissue. It was hypothesised that the presence of serum proteins may have interfered with the polyphenol inhibition of VEGF-induced VEGFR-2 phosphorylation, given the knowledge that polyphenols are able to bind to

a variety of biological proteins (Zinellu et al., 2014, Ozyurt et al., 2016, Soares et al., 2007). However, considering the aortic ring model required FCS for growth and extraction of enough protein from the aortic rings to analyse VEGFR-2 phosphorylation would require a large amount of tissue, a simpler model of VEGF-induced VEGFR-2 phosphorylation was needed. Therefore, the HUVEC model, as reported in chapter 2, was ideal for investigation of the effect of FCS on polyphenol inhibition of VEGF-induced VEGFR-2 phosphorylation.

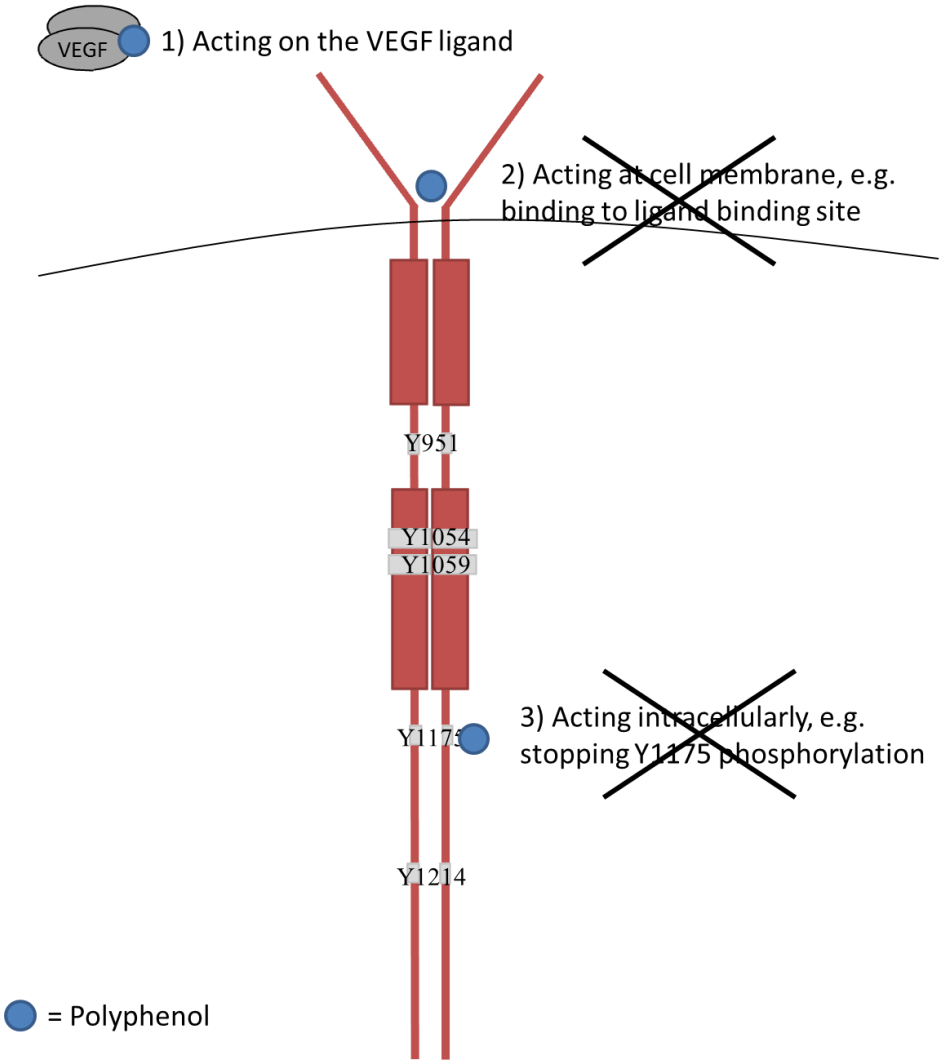

Figure 5.1: Diagrammatic representation of the mechanism of action for polyphenol inhibition of VEGF signalling reported in this thesis

4.5.2 Effect of FCS on polyphenol inhibition of VEGF signalling after 5 minutes

In the HUVEC model EGCG and EA were both able to strongly inhibit VEGF-induced VEGFR-2 phosphorylation at 1 µM independently and in combination. For both polyphenols, independently and in combination, this inhibitory effect was completely lost when the polyphenol was added to the VEGF containing treatment already supplemented with FCS. Interestingly, when the VEGF and EGCG were pre-mixed for 5 minutes prior to the addition of the FCS, the VEGF remained completely inactive, at least at this 5 minute time point. This suggests that the FCS was unable to affect the interaction between the VEGF and EGCG, which caused the inhibition of VEGFR-2 phosphorylation, after this 5 minute incubation (Figure 4.5). When ellagic acid was pre-mixed with VEGF prior to the addition of FCS significantly more VEGF-induced VEGFR-2 phosphorylation was observed (28 %) than the VEGF + EA only treatment after only 5 minutes. This demonstrates that VEGF regains significant VEGFR-2 phosphorylating activity after only 5 minutes incubation of the VEGF/EA complex with FCS. However, it is of note that a significant amount of inhibition is still retained (see Figure 4.6). Without further time points investigating EA inhibition alone we cannot conclude whether VEGF would regain more activity if the VEGF/EA complex was incubated for a longer time in the presence of FCS.

This difference between the inhibition capabilities of VEGF by EGCG and ellagic acid after 5 minutes incubation with FCS may be due to the binding strength of the interaction between the individual polyphenols and the VEGF ligand. While both EGCG and ellagic acid are predicted to interact within the ridge on the VEGF dimer where the VEGF receptor binding region sits, the binding affinity of EGCG for VEGF was higher than that for ellagic acid (change in Gibbs free energy -8.3 kcal/mol for EGCG versus -7.5 kcal/mol for ellagic acid) (Cerezo et al., 2015). In other experiments the binding constant, as a measure of affinity, of ellagic acid to HSA was determined as 8.47 ± 0.52 10^4 L/Mol (Tang et al., 2013) while EGCG has been recorded as having both a higher (Zinellu et al., 2014) and lower (Ozyurt et al., 2016) binding constant to HSA than ellagic acid (5.81 10^4 L/Mol and 11.0 10^4 L/Mol). However, on average, EGCG had a similar binding constant to HSA as ellagic acid.

Taking into account that data for both compounds on their ability or predicted ability to bind to both VEGF and serum albumin it could be suggested that, while the ability of both polyphenols to bind to serum albumin may be comparable, EGCG maintains a stronger binding affinity to VEGF than ellagic acid. Therefore, ellagic acid may be more likely to dissociate from VEGF in the presence of serum albumin than EGCG which would explain why EGCG was able to inhibit VEGF signalling in the presence of FCS after 5 minutes while VEGF regained 28 % activity following pre-mixing of the VEGF with ellagic acid under the same conditions. When EGCG and ellagic acid were used in combination the results obtained mirrored those achieved in the EGCG only experiments demonstrating that there is no impact of the polyphenol combination on the inhibitory capabilities of EGCG in the presence of FCS after 5 minutes.

4.5.3 Effect of FCS on polyphenol inhibition of VEGF signalling over longer duration

In further experiments the effect of FCS on EGCG and ellagic acid combined inhibition of VEGF-induced VEGFR-2 phosphorylation over time was investigated. This provided a greater understanding about the strength of interaction between the polyphenols and the VEGF ligand but could also act as an aid to interpretation of the results observed in the animal intervention study. Investigating the activity of the VEGF in the presence of FCS over time when the VEGF and polyphenol have been pre-mixed would provide insight into whether the inhibited VEGF of VEGF/EGCG/EA injection treatment arm from the study in chapter 3 would regain activity over a longer duration in a protein rich environment. It was clearly demonstrated that VEGF regained some ability to stimulate VEGFR-2 phosphorylation over time with a significant increase in VEGFR-2 phosphorylation in the 'VEGF/EGCG/EA + FCS' treatment after 4 hours of incubation at 37 °C. This suggests that a protein rich environment, in this model FCS supplementation to the media, can interfere with the potent inhibition of VEGF by EGCG and ellagic acid and that the strength of this interference is dependent on time. It is important to note that over 2/3 of VEGF activity remained inhibited after 4 hours and without further time points we cannot conclude whether the VEGF would regain more

activity, although the trend of the data suggests this may be the case. In the experimental arm from the study in chapter 3 where mice ate an EGCG and ellagic acid supplemented diet the polyphenol would enter an already protein rich environment, the blood, before it would elicit its anti-VEGF effects. Therefore investigating the capability of the polyphenols to inhibit VEGF-induced VEGFR-2 phosphorylation after addition to a serum protein rich environment over time would provide information on whether a polyphenol could elicit inhibitory effects after dietary supplementation. The VEGFR-2 phosphorylation activity of VEGF in the 'VEGF/FCS + EGCG/EA' treatment was reduced over time however given the instability of VEGF in culture media (Figure 4.9), the loss of VEGF stimulated VEGFR-2 phosphorylation could simply be an artefact of VEGF instability or degradation and not the polyphenol exerting an inhibitory effect. In future studies quantifying the VEGF protein in the media at the time of treatment could provide an insight into whether the observed effects were indeed just VEGF degradation or evidence of the polyphenol being able to elicit an inhibitory effect after a longer incubation.

The addition of FCS and polyphenols to the media increased the VEGF stability over the 4 hour period investigated. While it has previously been demonstrated that increased stability of VEGF correlates with serum protein concentration there is no previous literature describing the binding of VEGF to serum albumin. In mice genetically altered to express recombinant human VEGF the yield of the VEGF protein was higher when human serum albumin was co-expressed (Baur et al., 2005) suggesting that the albumin in the FCS could play a role in the increased stability of VEGF observed in these experiments. It is also known that heparin can be found in the circulating blood and therefore in serum (Engelberg, 1961). Assuming that the interaction of VEGF with other protein would increase VEGF stability heparin is also a known VEGF binding partner (Ashikari-Hada et al., 2005) and perhaps increased the stability of the VEGF in the media. However, it has been shown previously that VEGF can be sequestered from biospheres depending on the serum concentration of the solution but this was not associated with serum-borne heparin (Belair et al., 2014) suggesting the VEGF may have an affinity for something else in the serum. Interestingly the ability of VEGF to bind to a synthetic binding

partner *in vitro* was also affected by serum protein concentration. This reduction in binding was only demonstrated with generalised serum proteins and did not occur when an equivalent concentration of pure albumin was used, suggesting other proteins present in serum were able to interfere with VEGF binding to its binding partner *in vitro* (Belair and Murphy, 2013). Given that the half-life of VEGF has been extended through the addition of a mixture of FCS, EGCG and EA to the media, and given that binding of protein therapeutics to a variety of serum proteins has been shown to extend the half-life *in vivo* (Kontermann, 2011) it appears possible that the FCS proteins could cause this increased longevity effect. Furthermore, if these effects are down to serum protein binding to VEGF perhaps the serum proteins may be able to interfere with VEGF binding to the polyphenol. This could explain the lack of observed polyphenol inhibition of VEGF signalling when the VEGF and FCS are present in the media together prior to polyphenol addition. However, as stated before, there is no data at present which demonstrates that VEGF can bind serum proteins so further investigation into this would be required. It must also be recognised that the polyphenols could be causing this increased longevity of the VEGF protein. Polyphenol-protein interactions between ferulic acid with BSA (Ojha et al., 2012) and caffeic acid with milk proteins (O'Connell and Fox, 1999) have increased the thermal stability of the proteins. It is possible that this may account for the sustained VEGF-induced VEGFR-2 phosphorylation observed in the 'VEGF/FCS + EGCG/EA' treatment over the VEGF only treatment. However, if free polyphenol was able to bind to VEGF to increase its stability it would be logical to assume the VEGF activity would also be inhibited.

Another explanation for the lack of EGCG and ellagic acid inhibition in the presence of FCS is the interaction of serum proteins and the polyphenols (Ozdal et al., 2013). Human serum albumin has been shown to increase the stability of polyphenols such as the green tea catechins which are usually highly unstable in an aqueous environment (Zinellu et al., 2015), with this effect attributed to the binding of polyphenols to the HSA (Zinellu et al., 2014). Equally, as described, a large range of polyphenols are known to interact with serum albumin with EGCG and ellagic acid demonstrating reasonable affinity (Skrt et al., 2012, Bourassa et al., 2010, Dobreva

et al., 2014, Zinellu et al., 2014, Tang et al., 2013). A number of papers discuss the binding of polyphenols to serum proteins such as albumin as a mechanism by which stability and solubility issues in circulation are overcome (Bae et al., 2009, Pantusa et al., 2012). However, the literature does not provide an explanation or demonstration for how the polyphenols are able to be released from this binding and whether they are able to elicit their activity at the required locations. The data presented here in this chapter is therefore important as it demonstrates that the serum proteins prevented the inhibitory activity of the polyphenol. In the context of the animal study the data presented in this chapter presents a plausible suggestion that the polyphenols used in the intervention were inactive, at least in the capacity of inhibiting VEGF-induced VEGFR-2 phosphorylation, due to interference from serum proteins. It is important to remember that the strength of binding of polyphenols for serum albumin appears to be species specific (Zinellu et al., 2014) so caution must be taken as calf serum, and not mouse serum, was used in the data presented in this chapter.

Considering the potency of inhibition of VEGF-induced VEGFR-2 phosphorylation by EGCG and EA in serum free media and comparative lack of inhibitory activity in the presence of serum proteins, another important discussion point is raised. The majority of research into the biological effects of polyphenols has been undertaken in serum-free media conditions, typically to minimise variation between serum batches or for cell cycle synchronisation, however this can result in a different cellular response to treatment in comparison to non-serum starved cells. For examples, serum-starved HUVECs have been demonstrated to be more vulnerable to pro-inflammatory insult in comparison to HUVECs maintained in higher serum media (Russell and Hamilton, 2014). In another investigation FCS inhibited LPS-induced nitric oxide and TNF-α expression (Ohki et al., 1999). Considering observations such as these and the impact that serum proteins had on the inhibitory activity of polyphenols as demonstrated in this chapter it is important that further investigation of the relationship between biological proteins and polyphenols, and the impact this can have on the bioactivity of the polyphenols is undertaken. Furthermore, it would be prudent to use more biologically relevant cell

models when investigating the biological activity of polyphenols to determine the biological effect, before proceeding to animal intervention trials.

4.6 Conclusions

The data presented in this chapter shows that 1) the potent inhibitor of VEGF induced VEGFR-2 phosphorylation in serum-free media, EGCG, was not able to inhibit VEGF induced angiogenesis under the protein rich conditions of the aortic ring assay; 2) the presence of FCS in the treatment media containing VEGF prevented EGCG and ellagic acid inhibition of VEGF-induced VEGFR-2 phosphorylation in HUVECs 3) Addition of FCS to the treatment media resulted in the VEGF activity being slowly recovered following complete inhibition of VEGF-induced VEGFR-2 phosphorylation by mixing with EGCG and ellagic acid. This data may provide one explanation for the lack of inhibition of atherosclerosis progression by EGCG and ellagic acid observed in chapter 3, considering the expected upregulation of endogenous VEGF previously observed in atherosclerotic plaques. Taken into the wider context of polyphenol research, this demonstrated the difference in research outcomes that can occur in a cellular experiment in the presence or absence of FCS and how the biological activity of polyphenols may be misinterpreted in experiments using serum-free media. It also suggests that the effects of serum proteins should be considered when designing an experiment investigating the biological effects of polyphenols and that using a more biologically relevant cell culture model may provide improved insight into the biological effects of polyphenols.

Chapter 5

Discussion

5.1 Summary of main findings

The overall aims of this PhD project were to 1) to investigate if and how dietary polyphenols could inhibit VEGF induced VEGFR-2 phosphorylation in human umbilical vein endothelial cells, 2) to determine whether the polyphenols which elicit potent inhibition of VEGF induced VEGFR-2 phosphorylation could, in turn, inhibit VEGF induced atherosclerosis. The main findings from the thesis are summarised below:

- The most abundant green tea and pomegranate polyphenols, EGCG and punicalagin, are potent and rapid inhibitors of VEGF-induced VEGFR-2 phosphorylation in HUVECs. Metabolism of the pomegranate polyphenol punicalagin to ellagic acid, and further metabolism of ellagic acid to the urolithin and urolithin glucuronide compounds reduces the efficacy of the polyphenols to inhibit VEGF-induced VEGFR-2 phosphorylation in HUVECs.

- EGCG and ellagic acid were able to effectively inhibit VEGF-induced VEGFR-2 phosphorylation at physiologically relevant concentrations. Urolithin C was able to significantly inhibit VEGF induced VEGFR-2 phosphorylation at a concentration close to that previously observed in plasma.

- The polyphenols EGCG, punicalagin, ellagic acid, urolithin D and urolithin C inhibited VEGF-induced VEGFR-2 phosphorylation primarily through interaction with the VEGF ligand and not actions on the cell or receptor.

- 3 once weekly intraperitoneal injections of VEGF at 2 μg/kg body weight did not stimulate atherosclerotic plaque growth above a serum albumin injected control in the ApoE-/- mouse model fed an atherogenic diet after 6 weeks.

- 6 week diet supplementation with 3.4 mg EGCG and 4.3 mg ellagic acid per mouse per day in the ApoE-/- mouse model fed an atherogenic diet did not reduce the size of the atherosclerotic plaque in comparison to mice fed the

diet without polyphenols. A small but significant increase in plasma LDL and total cholesterol was seen in the polyphenol fed mice.

- The polyphenols EGCG and ellagic acid were unable to inhibit VEGF-induced VEGFR-2 phosphorylation in HUVECs in 2 % foetal calf serum supplemented media

5.2 Do higher circulating VEGF levels increase pathological angiogenesis?

It has previously been reported that a single intraperitoneal injection of VEGF at 2 µg/kg body weight was able to significantly increase atherosclerotic plaque size in both a hypercholesteraemic mouse model (ApoE-/-ApoB^{100}-/-) and a high cholesterol fed New Zealand white rabbit model. The repeatability of this observation in two different species suggests that VEGF induction of atherosclerosis is not dependent on genetic hypercholesterolemia or species (Celletti et al., 2001a, Celletti et al., 2001b). However, another study looking at the effect of adenoviral VEGF gene transfer in different mouse models of atherosclerosis found that only the mice with an ApoE-/- genotype (ApoE-/- and ApoE-/-LDLR-/-) had high plasma VEGF levels after gene transfer and were susceptible to VEGF induced atherosclerotic progression. The LDLR-/- and LDLR-/-ApoB$^{100/100}$ mouse models did not have increased VEGF plasma levels or aortic atherosclerosis following adenoviral VEGF gene transfer (Heinonen et al., 2013), suggested to be due to ApoB100-containing lipoprotein (increased in circulation in LDLR-/- mice) upregulation of VEGFR-1 expression by which acts as a sink for VEGF-A thereby limiting its stimulatory activity (Avraham-Davidi et al., 2012). This suggests that while the VEGF induction effect is not species specific, that changes in lipid-regulation can alter the VEGF response. However, the ApoE-/- model used in the study presented in chapter 3 was previously reported to be susceptible to VEGF-induced atherosclerosis following adenoviral VEGF gene transfer. Taking this into account, the ApoE-/- mouse used in chapter 3 appears to be an appropriate model for this type of investigation.

Celletti et al. did not measure whether the VEGF was active at the time of injection although the increase in atherosclerotic plaque size was specific to the VEGF injection only which strongly suggests that it was. In the study presented in chapter 3 it was demonstrated that the VEGF in the injection solution was active and able to stimulate VEGFR-2 phosphorylation in HUVECs. However, one limitation of both studies is that the VEGF concentration in plasma following VEGF intraperitoneal injection was not determined. MSA has been demonstrated to reach circulation following intraperitoneal injection (Kijanka et al., 2014) but as yet the same has not been reported for VEGF. The decision to not measure VEGF in plasma was taken as the time for VEGF to reach the plasma is unknown therefore a time course post injection would be required and considering the strong stimulatory effects of VEGF observed previously that this would be an excessive use of animals. However, given the lack of observed effect of VEGF stimulation of atherosclerosis progression in the study presented in chapter 3, even after including a further 2 VEGF injections compared to the Celletti et al. paper, it would be prudent to investigate the concentration of VEGF in mouse plasma over time following a single intraperitoneal injection of 2 µg/kg body weight to determine whether VEGF is able to pass into the plasma at a significant concentration.

Another point for consideration is whether the VEGF, upon reaching the plasma, was active as the data presented in this thesis did not address this question. Previously 2 out of 7 serum proteins were found to undergo modification prior to absorption from the peritoneal cavity following injection (Regoeczi et al., 1989) however no literature to date discusses stability or activity of VEGF following absorption from the peritoneal cavity. Higher circulating VEGF levels positively correlate with increased vascularisation of the retinal epithelium in proliferative retinopathy (Lip et al., 2000), and therefore retinal angiogenesis may be a good method of quantification of systemic angiogenesis as a measure of VEGF activity in response to the VEGF injections (Stahl et al., 2010). The retinal tissue was fixed in formaldehyde at the time of animal death and therefore would be suitable for use in investigations into systemic angiogenesis in this study.

It was concluded by Celletti et al. that a single intraperitoneal injection of VEGF stimulated growth of the atherosclerotic plaque, at least in part, due to the upregulation of $CD34^+/Flk\text{-}1^+$ bone marrow cells and macrophage infiltration into the lesion (Celletti et al., 2001a, Celletti et al., 2001b). This was not measured in the study presented in chapter 3 and might provide insight into whether the VEGF was able to produce the same physiological response as reported previously. Macrophage infiltration and $CD34^+$ cell content in the atherosclerotic plaque can easily be measured although increased expression of $CD34^+/Flk\text{-}1^+$ hematopoietic progenitor cells in bone marrow cannot be quantified and bone marrow was not isolated from the mice in the chapter 3 study.

Atherosclerosis is a complex disease and the morphology of the plaque can contribute to the pathology of the disease without necessarily altering the size of the atherosclerotic plaque. For example, VEGF and blood vessel generation is associated with vulnerable plaque, fibrous cap thinning, and thrombosis. Vulnerable plaques are associated with an increased cardiovascular event risk such as stroke (Carter et al., 2007, Chen et al., 1999, Juan-Babot et al., 2003, Fleiner et al., 2004). Therefore if plaque instability is increased through rapid proliferation of immature blood vessels stimulated by the VEGF injections then the potential risk of atherosclerosis related death would be increased. To investigate this, the aortic plaque can be classified into disease stages as identified previously (Insull, 2009, Stary et al., 1995) or particular markers of unstable plaque such as high microvessel density, high macrophage content, large lipid core and thin fibrous cap can be stained for. This would provide an insight into whether the VEGF injections increased the cardiovascular risk without altering the plaque size.

Given that the ApoE-/- has been shown to be an appropriate model of VEGF induced atherosclerosis in the right conditions previously (Heinonen et al., 2013, Leppanen et al., 2005), the protocol used for injection mirrored that used by Celletti et al. (Celletti et al., 2001a, Celletti et al., 2001b), two further VEGF injections were undertaken than in the Celletti et al. studies, and that the VEGF was active upon injection support the conclusion that VEGF intraperitoneal injection in the ApoE-/- does not induce progression of atherosclerosis. However, investigation into the

plaque morphology and systemic angiogenesis in the study presented in chapter 3 would help interpret these results further.

5.3 Can polyphenols that inhibit VEGF signalling by interacting with VEGF *in vitro* inhibit pathological angiogenesis by binding to VEGF *in vivo*?

For the polyphenols capable of inhibiting VEGF-induced VEGFR-2 *in vitro* to be effective *in vivo* it would be expected that: 1) A dose capable of eliciting inhibition of VEGF-induced VEGFR-2 phosphorylation would have to be within the physiologically achievable range; 2) The inhibitory action would need to be effective under physiologically relevant conditions; and 3) it would need to be shown to inhibit VEGF-induced pathological angiogenesis, for example atherosclerosis, in an animal model.

In chapter 2 it was reported that the primary green tea and pomegranate polyphenols, EGCG and punicalagin, were both potent inhibitors of VEGF-induced VEGFR-2 with the concentrations required to inhibit 50 % phosphorylation (IC_{50}) being, 96 and 49 nM respectively. The bioavailability of the polyphenols is an important consideration when determining whether it is possible for the same inhibitory effect to occur *in vivo.* EGCG is found in circulation predominantly as an aglycone (Ullmann et al., 2003, Chow et al., 2001, Meng et al., 2002, Lee et al., 2002) and up to concentration of 700 nM (Henning et al., 2005) demonstrating that EGCG has the potential to reach an effective dose *in vivo*. However, punicalagin is thought to be very poorly bioavailable and not to reach the plasma (Mertens-Talcott et al., 2006) and is instead rapidly converted to ellagic acid early in the gastrointestinal tract (Gonzalez-Barrio et al., 2010). Ellagic acid is found in the plasma following pomegranate consumption up to 200 nM although, when given as an ellagic acid dose, higher plasma concentrations of over 600 nM were observed (Hamad et al., 2009). Under the model conditions presented in chapter 2 ellagic acid had an IC50 for inhibiting VEGF-induced VEGFR-2 phosphorylation of 310 nM suggesting that a concentration capable of inhibiting VEGF activity *in vitro* could be achieved *in vivo*. Considering that ellagic acid is also rapidly metabolised by the

microbiota to generate higher plasma concentrations of the urolithins (µM) (García-Villalba et al., 2013) the biological activity of these compounds on VEGF-induced VEGFR-2 phosphorylation also needed consideration. In chapter 2 the *in vitro* anti-VEGF activity of the urolithin microbial metabolites was investigated for the first time. The IC50 values for inhibition of VEGF-induced VEGFR-2 phosphorylation by urolithin D, C, A and B were 0.2, 3.7, 54.0, 251 µM respectively. This data demonstrates that each step of metabolism to remove a hydroxyl group from the urolithin compounds reduces the inhibitory capacity in this assay. Furthermore urolithin A and B glucuronide were unable to inhibit VEGF-induced VEGFR-2 phosphorylation at concentrations up to 150 µM suggesting that phase-II metabolism can also have a negative effect on this activity. This being said, the peak urolithin plasma concentrations following a high dose of ellagitannins or ellagic acid has not been undertaken and the highest reported urolithin C plasma concentration was 1.8 µM (Cerda et al., 2004), close to the 2 µM concentration which caused significant inhibition of VEGF-induced VEGFR-2 phosphorylation *in vitro*. Taking all data into account EGCG and ellagic acid are the only compounds capable of inhibiting VEGF-induced VEGFR-2 phosphorylation at concentrations found in plasma following dietary intervention.

The data presented in chapter 2 also reported that the polyphenols capable of inhibiting VEGF-induced VEGFR-2 phosphorylation did so predominantly through interaction with the VEGF ligand and not the cell. EGCG and urolithin C were completely ineffective at inhibiting VEGF-induced VEGFR-2 phosphorylation without coming into contact with the VEGF ligand while punicalagin, ellagic acid, and urolithin D were only able to elicit around 10 % phosphorylation inhibition through actions on the cell or receptor at the concentrations tested. Therefore for the compounds which reach and effective dose *in vivo*, EGCG and EA, to potently inhibit VEGF signalling *in vivo* the polyphenols would need to interact with the VEGF ligand in the blood. The *in vitro* work in chapter 2 was carried out in serum free basal media, as has been done previously (Huang et al., 2012, Lamy et al., 2006, Wen et al., 2008, Lamy et al., 2002, Lee et al., 2004, Liu et al., 2008, Neuhaus et al., 2004, Rodriguez et al., 2006, Lu et al., 2008, Shimizu et al., 2010, Pan et al., 2011, Cerezo

et al., 2015, Moyle et al., 2015). The addition of serum to the HUVEC model of VEGF-induced VEGFR-2 phosphorylation as presented in chapter 4 investigated this inhibition in a more physiologically relevant environment. It was found that the addition of 2 % FCS in the VEGF supplemented media prior to the addition of the polyphenol completely prevented the inhibitory action of the polyphenol. Furthermore, if the VEGF and polyphenol were pre-mixed in basal media before the addition of FCS then gradually over time the presence of the serum mediated the recovery of VEGF activity. In healthy human serum total protein values are 60 – 80 g/L (Kyle and Greipp, 1978, Gahutu and Wane, 2006). Around 50 g/L total serum proteins are found in plasma from C57BL6 mice while in ApoE-/- strains the total serum protein level is slightly elevated above the standard laboratory mouse at 57 g/L (Zaias et al., 2009). The literature suggests that both EGCG and ellagic acid have an affinity for serum albumin (Ozyurt et al., 2016, Zinellu et al., 2014, Tang et al., 2013) and given that the protein content in 2 % serum supplemented media will be well below the protein content in the blood it can be concluded that EGCG and ellagic acid are unlikely to inhibit VEGF activity *in vivo* by interacting with the VEGF ligand given the presence of a high serum concentration in plasma.

In chapter 3 it was reported that dietary intake of EGCG and ellagic acid at 3.4 and 4.3 mg/mouse/day respectively does not reduce atherosclerosis in the ApoE-/- mouse fed an atherogenic diet. The equivalent dose for a 60 kg human would be around 770 mg EGCG and 970 mg ellagic acid daily (Reagan-Shaw et al., 2008), a dose only typically achievable with dietary supplements. However, considering there was no-stimulation of atherosclerosis progression by the VEGF injections it is difficult to conclude the effect of EGCG and EA dietary intervention at these doses on VEGF-induced atherosclerosis. However, it is known that VEGF and VEGFR-2 are frequently reported to be upregulated in those suffering with atherosclerosis and in atherosclerotic tissue (Ho-Tin-Noe et al., 2011, Kimura et al., 2007, Blann et al., 2002, Fleisch et al., 1999, Kucukardali et al., 2008) with many morphological features of the atherosclerotic plaque such as macrophage uptake of oxidised LDL known to upregulate VEGF protein release (Riazy et al., 2009). Therefore, if it could be identified if VEGF and VEGFR-2 were upregulated over time in the

atherosclerotic tissue and mouse plasma collected from the study presented in chapter 3 (for example at week 3 and/or week 6 over baseline) irrelevant of injection type it would provide information as to whether the polyphenols were able to inhibit the upregulation of endogenous VEGF signalling associated with atherosclerosis. Unfortunately, due to time restrictions, these investigations were not able to be completed as part of this thesis. Therefore, the data presented in this thesis support the notion that it is unlikely EGCG and EA could inhibit VEGF-induced pathological angiogenesis through interactions with the VEGF ligand in a serum protein rich environment such as blood. However, it does not limit the possibilities for alternative methods of polyphenol treatment for example topical application or intravitreal injection of polyphenols.

5.4 What are the wider implications of the influence of serum proteins to the field of polyphenol research?

Polyphenol have been reported to inhibit numerous key cell signalling pathways through inhibiting phosphorylation events. This includes a large body of work with one of the main polyphenols investigated in this thesis, EGCG. Many papers report polyphenol effects in serum-free or low serum media experiments, primarily due to serum-starving to normalise the cell population or to reduce variables. In previous experiments delphinidin (Lamy et al., 2006, Teller et al., 2009), EGCG (Lamy et al., 2002), and quercetin (Pratheeshkumar et al., 2012) were reported to inhibit VEGFR-2 phosphorylation through actions on the VEGF receptor and not the ligand after extended incubation periods were undertaken in serum-free media. Treatment with ellagic acid and VEGF in serum-free media was also reported to reduce VEGFR-2 phosphorylation, *in vitro* tyrosine kinase activity and microvessel generation from aortic rings (Wang et al., 2012). Polyphenol inhibition of other receptors is also investigated in serum-free media for example, in decreasing potency, delphinidin, quercetin and EGCG in combination with epidermal growth factor treatment inhibited phosphorylation of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/ErbB2) (Teller et al., 2009, Fridrich et al., 2008). Similarly green and black tea polyphenols, specifically theaflavin monogallate and epigallocatechin digallate, were able to act as competitive inhibitors of ephrin

(Eph) to reduce EphA4 receptor phosphorylation in serum-free media (Noberini et al., 2012). Where serum is used it is typically at low concentrations. For example, apigenin and luteolin were also found to inhibit platelet derived growth factor receptor β (PDGFR-β) phosphorylation in 0.5 % BSA containing media (Lamy et al., 2008). While serum proteins were present in this model they were at much lower concentrations that would be encountered *in vivo* (Kyle and Greipp, 1978, Gahutu and Wane, 2006, Zaias et al., 2009). Physiological responses to polyphenols have also been reported in serum-free studies. EGCG inhibition of oral epithelial cell growth was also markedly reduced in the presence of serum proteins in comparison to basal media, demonstrating others have observed this effect previously (Hong et al., 2009). Considering the observation in chapter 4, it is logical to hypothesise that the presence of serum-proteins in these models would have the potential to affect the previously reported actions of polyphenols. However, there are many papers in the published literature which do not discuss the specific content of the media used to conduct the experiments. The data presented in this thesis highlights that this should be important considerations and further importance should be given to undertaking more physiologically relevant cell based experiments, such as those with higher serum content. However, this would require a wider awareness of the impact of serum and biological proteins on polyphenol activities *in vitro*.

Furthermore cell culture investigations into the mode of action of the polyphenol on VEGF-induced VEGFR-2 phosphorylation inhibition could be applied more widely to provide greater understanding to other inhibitory actions reported. Taking delphinidin, quercetin and EGCG inhibition of EGF-stimulated EGFR phosphorylation as an example, the study design provided the opportunity for the polyphenols to interact with both the receptor and ligand (Fridrich et al., 2008, Teller et al., 2009). Utilising a side-by-side comparison of polyphenol actions on the cell alone versus actions when used in combination with the ligand, as undertaken in chapter 2, could provide critical information as to where the polyphenols were exerting the most potent effects. Through the use of this side-by side approach it was identified in chapter 2 that for the polyphenols to potently inhibit VEGF signalling *in vivo* they would need to be able to interact with VEGF within a biological system at

physiological concentrations. If this approach could be taken to other signalling pathways to understand where and how the polyphenol may exert its effect we may be able to tailor the *in vitro* biological treatment to match the biological system where the *in vivo* effects would be predicted to occur.

5.5 Summary of recommendations for future work

5.5.1 Further interpretation of animal intervention in chapter 3

A key aim outlined in chapter 1 was to determine whether the polyphenols which elicit potent inhibition of VEGF-induced VEGFR-2 phosphorylation could, in turn, inhibit VEGF-induced angiogenesis-driven atherosclerosis. This relied on the induction of atherosclerosis by VEGF injection as previously reported (Celletti et al., 2001a, Celletti et al., 2001b). Below, 3 key further investigations into the tissues from the animal study in chapter 3 are outlined which would help to conclude 1) whether intraperitoneal injected VEGF is capable of inducing angiogenesis and atherosclerosis and 2) whether EGCG and ellagic acid dietary supplementation was able to alter alternative markers of atherosclerosis.

Firstly, concluding whether systemic angiogenesis was upregulated in the VEGF injected animals beyond the levels observed in the MSA injected animals would provide information as to whether the VEGF was active upon entry in to the blood system. One way to measure this would be with retinal angiogenesis, which is highly susceptible to increased circulating VEGF, as a marker of systemic angiogenesis levels (Stahl et al., 2010, Kliffen et al., 1997). This is undertaken by dissecting the retinal epithelium from the retinal pigment epithelium under a microscope and preparing the retinal epithelium for flat-mounting and staining with a blood vessel marker, for example isolectin B_4 (Stahl et al., 2010). Quantification can be undertaken by counting the branch points in a specified field of view (Stenzel et al., 2011). Retinopathy associated vascular tufts can also be quantified (Lamoke et al., 2011). Should an effect be observed in the VEGF injected animals the same measurement can be used to determine whether the polyphenols were able to inhibit systemic angiogenesis in the study.

Secondly, it would need to be determined if the VEGF injections, while they did not stimulate increases in plaque size, were able to alter the pathology of atherosclerosis as determined by morphological analysis. Increased VEGF expression is associated with increased plaque vulnerability and rupture (Chen et al., 1999), thereby increasing the risk of a cardiovascular event (Eliasziw et al., 1994). Therefore, this analysis may provide further insight into whether the VEGF injections caused a more dangerous pathology in the animals. Standard histopathological stains such as Masson's trichrome or Movat's pentachrome would identify all connective tissues (Movat, 1955). Immunohistochemistry staining for macrophage infiltration and fibrinogen, including to mark any locations where thinning of the fibrous cap has occurred are also important to help classify the stage of atherosclerotic disease (Rosenfeld et al., 2000). Large acellular necrotic regions and immunohistochemical staining for intra-plaque vessels with a vascular endothelial marker such as von Willebrand's factor (vWf) are crucial. Perivascular diffusion of vWf into the surrounding tissue also gives an indication to vessel leakage (van Lammeren et al., 2012, Virmani et al., 2005). Should a difference be observed between the VEGF injected mice versus the serum albumin injected mice this analysis can be undertaken within the aortic cryosections from the polyphenol-fed mice to determine if the EGCG and ellagic acid supplemented diet was able to affect plaque stability or atherosclerotic disease classification in the ApoE-/- mouse.

Finally, it should be determined if endogenous VEGF signalling was upregulated in the plaque irrelevant of injection type. This can be done by comparing the plaque between the time points instead of treatments. VEGF and VEGFR-2 expression in the plaque can be measured by immunohistochemical staining with anti-VEGF and anti-VEGFR-2 antibodies. Alterations to VEGF or VEGFR-2 protein or gene expression could also be analysed in the lung tissue as it is a rich source of VEGF expressing vascular endothelial cells (Berse et al., 1992). Investigating the circulating VEGF levels in the mouse plasma may be difficult as the limit of detection is 9 pg/ml (Manenti et al., 2005) however VEGF ELISA analysis (Keyes et al., 2003) of the mouse plasma can be undertaken to indicate whether changes to

circulating VEGF levels have occurred in response to any treatments within the animal study presented in chapter 3.

Should these investigations into the remaining tissue from the animal study in chapter 3 demonstrate that VEGF was not active, did not induce morphological changes in the plaque and there was not an endogenous upregulation of VEGF It can be concluded that in this experiment VEGF injection did not induce atherosclerosis as has been observed previously (Celletti et al., 2001a, Celletti et al., 2001b). However, further small-scale animal studies into VEGF concentration in plasma over time following a single 2 μg/kg body weight VEGF injection would also provide information on the pharmacokinetics of VEGF entry into the plasma while labelled VEGF could be injected into the animal and with the appropriate scanner the dissemination throughout the body could be tracked in real-time as has been done previously following intravenous VEGF injection (Wang et al., 2009). This would provide further information as to whether the VEGF can reach the target tissues following intraperitoneal injection.

5.5.2 Alternative animal intervention trials to investigate hypothesis

Given that it was previously observed that adenoviral gene transfer of human VEGF into the ApoE-/- mouse model resulted in induction of atherosclerosis (Heinonen et al., 2013) the dietary intervention study with EGCG and ellagic acid could be repeated using this model. However, considering the implications of the presence of serum on the VEGF inhibitory activities of these polyphenols perhaps intervention with alternative VEGF inhibitory polyphenols could be undertaken, for example the apple procyanidins (Moyle et al., 2015), following investigation into their inhibitory effects in the presence of serum proteins. Furthermore, utilising VEGF adenoviral gene transfer in other models of atherosclerosis where progression of atherosclerosis is not so accelerated, for example wild type mice or New Zealand white rabbits fed a high cholesterol diet, could provide a more natural model of VEGF induced atherosclerosis to provide insight into the morphology of VEGF-associated atherosclerotic disease. Retinal vascularisation could be used as a measure of systemic angiogenesis across the study using live imaging equipment to

visualise retinal angiogenesis without the need to sacrifice animals at each time point (Nakao et al., 2013).

It would also be interesting to undertake further investigations into the *in vivo* effects of EGCG and ellagic acid on VEGF signalling using alternative routes of polyphenol delivery. For example, currently anti-VEGF monoclonal antibody treatments are injected into the vitreous of the eye but there are side effects such as increased risk of thrombotic events (Thulliez et al., 2014). The protein content in the eye is much lower than the plasma at around 1-4 g/L (Skeie et al., 2012) although are slightly raised in VEGF associated retinopathies such as proliferative diabetic retinopathy (Loukovaara et al., 2015). EGCG and ellagic acid could provide an interesting candidate for an alternative intravitreal injection therapy. EGCG and/or ellagic acid could be injected into the vitreous of the ischemia-induced retinopathy mouse model which has been used previously to demonstrate the anti-angiogenic potential of tyrosine kinase inhibitors (Maier et al., 2005). Equally, it has previously been demonstrated that topical application of EGCG to mouse skin reduced tumour initiation and growth by up to 87 % (Huang et al., 1992) and this effect was reported to be due to stopping the phosphorylation events associated with tumour progression. Given the advances in technologies since that publication a similar study could be conducted which measures blood vessel density and VEGFR-2 expression in the tumours *in vivo* as well as post-mortem histology of tissue to provide key insights into whether inhibition of VEGF signalling is responsible for this observation. Ellagic acid could also be an interesting candidate for investigation into topical application to skin cancer to reduced pathological angiogenesis.

5.5.3 Further investigation into polyphenol-protein interactions

The data presents an interesting avenue of investigation into the interactions between polyphenol and biologically relevant proteins. Investigating the nature of these interactions would provide key information to determine whether *in vitro* observations can be translated into a physiological environment. One example of such an experiment would be saturation transfer difference (STD) NMR, where the spin polarisation of the small molecule particles (e.g. polyphenol) is altered through

interaction with a protein (e.g. human serum albumin) and this change can be detected can providing information about location and strength of the interaction. This has previously been used to characterise the binding of tryptophan to human serum albumin (Viegas et al., 2011). Another method which may be useful is high performance affinity chromatography (HPAC) using a column containing immobilised serum albumin as the stationary phase would provide information on the binding kinetics of the protein-polyphenol interaction. Competitive displacement assays can be conducted, for example running VEGF in the mobile phase across the polyphenol bound to the serum protein stationary phase and measuring the concentration of polyphenol to be eluted (Hage et al., 2011). Surface plasmon resonance technology can detect very small binding or interaction events and could be useful for investigation of polyphenol-protein interactions. Taking the interactions between VEGF and polyphenols as an example, the VEGF ligand can be bound onto a Biacore surface plasmon resonance chip and the polyphenol can be run across the chip in solution in the presence and absence of serum proteins to compare the binding parameters. Similarly the FCS can be run through before and/or after the addition of the polyphenol to further investigate the strength of interaction. This would help interpret the observation shown in Figure 4.8 that polyphenol bound VEGF can regain activity in the presence of FCS over time. X-ray crystallography of a small molecule-protein complex can also be undertaken to identify the binding locations of a small molecule however there are many possible confounding factors with this route of experimentation and care should be taken to consider these complications (Cooper et al., 2011).

Finally, the data presented in this thesis and the discussion in section 5.4 suggests a general recommendation that future research takes into consideration the impact of serum or serum proteins on the biological activity of polyphenols. Comparison between the cellular responses to polyphenols in the presence or absence of serum or serum proteins will be important for understanding whether *in vitro* observations can be translated to *in vivo* observations. However, this would require the integration and appreciation of more biologically relevant cell culture experiments by researchers, reviewers, editors and the wider scientific community.

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