Application of zebrafish and murine models in lipoprotein metabolism and atherosclerosis research

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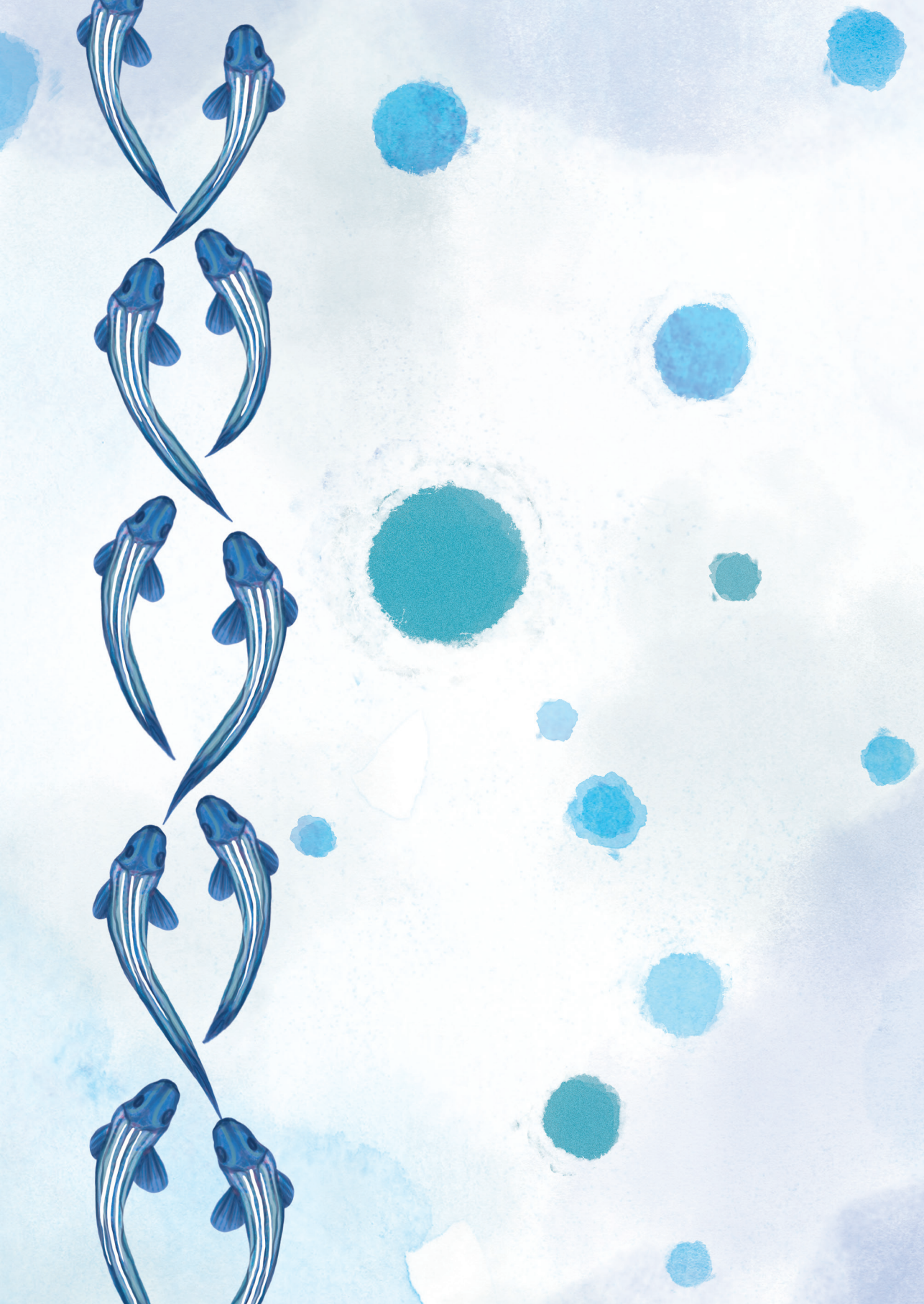

CHAPTER 2

ZEBRAFISH ATHEROSCLEROSIS: EXPERIMENTAL DEFINITIONS AND DIFFICULTIES

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Letter to the editor

To the editor,

Macrophages play an important role in atherogenesis. Early stages of the disease are characterized by the accumulation of foam cells, derived from macrophages that have taken up excessive amounts of cholesterol. Our lab studies the role of macrophages in atherosclerosis progression as well as regression. Mice represent the most widely used model organism for studying atherosclerosis. However, a clear limitation of using mice is that only end-point atherosclerosis measurements are possible. In this context, zebrafish provide an attractive alternative atherosclerosis model because of its optical transparency and the associated ability for non-invasive cell, i.e. macrophage tracking. With great interest, we have therefore read the paper of Yan *et al.* (2018), who recently established a zebrafish atherosclerosis model showing high cholesterol diet (HCD)-induced vascular lipid accumulation and co-localization of myeloid cells with lipids in the caudal vein of zebrafish larvae[1].

The experimental setup of Yan and colleagues was based on a paper of Stoletov *et al.* (2009) who, more than 10 years ago, for the first time described the possibility to induce early atherogenesis in zebrafish[2]. Stoletov *et al.* demonstrated that wild-type adult (3-month old) zebrafish are susceptible to develop hypercholesterolemia, e.g. increased total cholesterol levels, and fatty streaks in the dorsal aorta upon 8-12 weeks of 4% (w/w) HCD feeding. Interestingly, larvae of wild-type zebrafish (15 days post fertilization (dpf)) also developed vascular lipid deposits in the caudal vein after only 10 days challenge with fluorescently labelled 4% HCD. In their studies, Stoletov *et al.* used the *Lyz:DsRed2* transgenic zebrafish line to examine the effect of HCD on myeloid cell recruitment. Upon HCD challenge, more myeloid cells were recruited to the caudal vein, but the possible co-localization of these cells with the vascular lipid deposits was not determined. In this light, it should be noted that under control of the lysozyme promotor all myeloid cells are labelled and hence that it is not macrophage-specific[3]. Similarly, in the recently published manuscript Yan *et al.* the *Coronina1a:eGFP* line was used, which also labels all myeloid cells as well as T-lymphocytes[4].

To examine the specific role of macrophages in early atherogenesis in zebrafish larvae, we used a zebrafish line in which the *Mpeg1* promotor drives fluorescent transgene expression[5]. At five dpf, the 10 day-challenge with 4% HCD labeled with 10 ug/g cholesteryl ester bodipy FLC12 was initiated, as previously described[2] (**Fig. 1A**). Although adult zebrafish develop hypercholesterolemia upon long-term HCD feeding, we did not observe a difference in total body cholesterol levels in the zebrafish larvae

after 10 days on HCD (**Fig. 1B**). However, we did note compensatory downregulation of genes involved in cholesterol metabolism, i.e. HMG-CoA reductase (*hmgcr*; -47%; p=0.05) and the low-density lipoprotein receptor (*ldlra*; -85%; p=0.003). No effect was found on the expression of ATP-binding cassette cholesterol efflux transporters *abca1* and *abcg1* (**Fig. 1C**). Co-localization of macrophages with labeled cholesteryl ester was used as readout for foam cell formation (early atherogenesis). We incidentally found foam cells in the caudal vein of the *Mpeg1:RFP* larvae. However, the numbers were very low and did not appear to be different between the control (CTRL) and HCD group (**Fig. 1D**).

Although both Stoletov *et al.*[2] as well as Yan *et al.*[1] have shown that early atherogenesis can be modulated in zebrafish larvae, we have not been able to reproduce their findings. As we followed the same protocol as published (**Table 1**), it is hard to pinpoint the underlying reason for the differential effects. In agreement with our current findings, Yoon *et al.* (2013) also did not find accumulation of leukocytes in the caudal vein of zebrafish larvae after 10 days on HCD[6]. The differential outcomes amongst the different laboratories might due to small differences in general zebrafish husbandry affecting the zebrafish microbiome and thereby immunity of the host[7]. Alternatively, the definition of early atherogenesis applied might influence the outcome.

In mammalian atherosclerosis, macrophages generally make up the majority of myeloid cells present in the early atherosclerotic lesion. Our apparently negative results whilst using a macrophage-specific transgenic line as compared to more general myeloid transgenics could indicate that other myeloid cells drive early atherosclerosis in zebrafish larvae. In support, Bandaru *et al.* (2019) demonstrated that 5 days of HCD feeding induces co-localization of lipids with neutrophils in the caudal vein[8].

In conclusion, our data show that wild-type zebrafish larvae are not as susceptible to HCD-induced accumulation of macrophage foam cells in caudal vein as was previously suggested. With this letter we aim to underscore the importance of clearly defining atherosclerosis when studying a new animal experimental model, as the application of different definitions can affect the interpretation of the results.

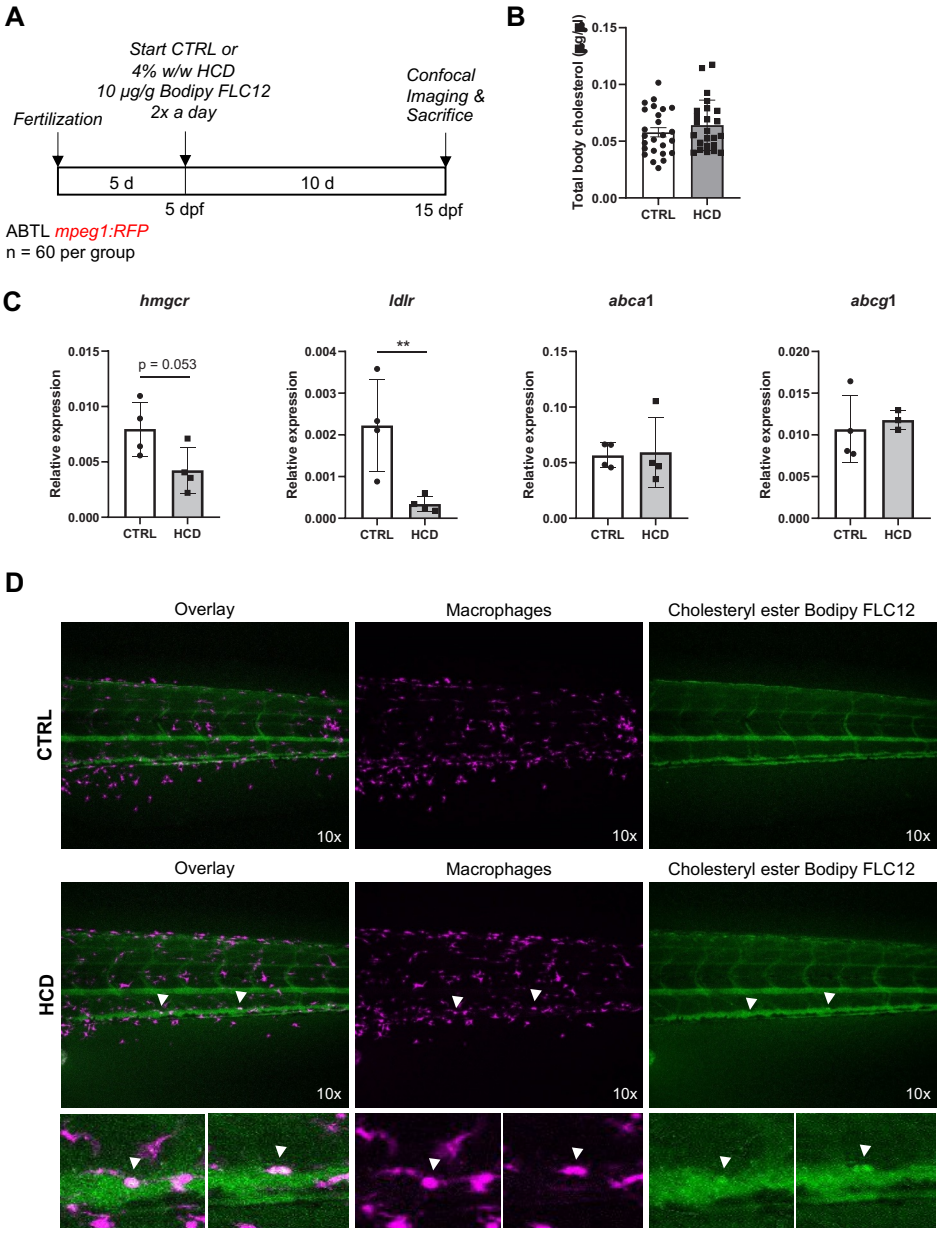

Figure 1. No evidence for enhanced macrophage foam cell formation in ABTL wild-type zebrafish larvae upon 10 days HCD feeding. (A) Experimental setup **(B)** Total body cholesterol levels (n=25) **(C)** Relative expression levels of the key genes involved in cholesterol metabolism (n=4 pools of 7 fish each) **(D)** Representative images of the caudal vein showing cholesteryl esters in green and macrophages in purple. Incidentally found foam cells, as identified by purple and green co-localization, are indicated by the white arrowheads. All data represent means ± SEM; $p<0.01$**

Table 1. Overview of the similarities and differences in setup between zebrafish larvae atherosclerosis studies. n/d = not described

	Current study	**Stoletov *et al.*[2]**
Tg zebrafish line	Tg*(Mpeg1:RFP)*	Tg*(Lyz:DSred2)*
Feeding	2 times a day 4% (w/w) HCD Standard dry food	- n/d 4% (w/w) HCD Artificial Artemia
Label	Cholesteryl ester analog 10 ug/g Bodipy FLC12	Cholesteryl ester analog 10 ug/g Bodipy 576/589 C11
Days of HCD	10 days	10 days

	Yan *et al.* [1]	**Yoon *et al.*** [6]	**Bandaru *et al.*** [8]
	Tg*(Coronina1a:eGFP)*	Tg*(Fli1:GFP)*	Tg*(Mpo:eGFP)*
	- n/d	- n/d	2 times a day
	4% (w/w) HCD	4% (w/w) HCD	4% (w/w) HCD
	- n/d	Artificial Artemia	Standard dry food
	Cholesteryl ester analog	Cholesteryl ester analog	Lipid-droplet marker
	10 ug/g Bodipy FLC12	10 ug/g Bodipy 576/589 C11	Monodansylpentane
	10 days	10 days	5 days

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