BioAnalytics

HISTOPATHOLOGY REPORT

Company: Texas Tech University
Study Director: Vijay Hegde
Study ID: E4orf1 as a novel anti-NAFLD agent
IDEXX BioAnalytics Case ID: 103046-2024
Date: 25/08/2024

Prepared by:

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Date: 2024.03.25 13:16:56 -05'00'

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Summary

Test article-related lower NASH liver score was identified in Group 2 – DR only, followed by Group 3 – E4 only, Group 5 – midpoint, and Group 4 – ED+DR when compared to Group 1 – Control. Fibrosis was identified in all Groups with similar frequency and severity. Special stain may help identifying more subtle difference in fibrosis across groups.

Atypical round cell infiltrates were observed in the liver of six of the eighteen mice and were interpreted as background findings (reactive lymphoid hyperplasia vs incipient lymphoma), possibly the result of the genetic mutation of these mice.

Inguinal adipose tissue was normal in all but two animals from Group 3 – E4 only where minimal histiocytic infiltrate was identified and one animal from Group 2 – DR only where minimal fibrosis was detected. There was no evidence of steatosis. The changes detected in the adipose tissue of these three animals were minimal and likely not clinically/biologically relevant/significant

Statement of Compliance

This histopathology study was not conducted in accordance with the principles set forth in the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations but was conducted in accordance with applicable Standard Operating Procedures (SOPs) of IDEXX BioAnalytics.

Introduction/History

Received by IDEXX BioAnalytics was a pool of tissues containing liver and inguinal adipose tissue from eighteen C57BL/6 from Texas Tech University, study E4orf1 as a novel anti-NAFLD agent, IDEXX BioAnalytics #case ID 103046-2024. The tissues had been fixed in 10% neutral buffered formalin. Samples belonged to four treatment groups, and a control set of animals was submitted. A detailed study protocol was not provided, but a general study overview was provided as follows:

In this current study we fed mice a high saturated fat, high fructose diet for 20 weeks to induce steatosis and fibrosis. We have fixed liver samples during end of study sacrifice and will need pathological evaluation for steatosis, hypertrophy, inflammation and fibrosis. We will send 12 mice (n=6 males and n=6 females).

This report aims to provide an interpretive summary of the histopathologic findings in the tissues of these mice.

Material and Methods

Received by IDEXX BioAnalytics was a pool of tissues containing liver and inguinal adipose tissue from eighteen C57BL/6 from Texas Tech University, study E4orf1 as a novel anti-NAFLD agent, IDEXX BioAnalytics #case ID 103046-2024. The tissues had been fixed in 10% neutral buffered formalin.

Samples belonged to four treatment groups and one, Group 1 - Control (n=3), Group 2 – DR only (n=3), Group 3 – E4 only (n=3), Group 4 – E4+DR (n=3), Group 5 - midpoint (n=6).

Submitted tissues were trimmed according to the RITA protocols (Registry of industrial toxicology animal -data/ https://reni.item.fraunhofer.de/reni/trimming/), processed for paraffin embedding followed by standard hematoxylin and eosin staining and followed by histopathologic evaluation.

Each slide was labeled with the provided study ID number, animal ID, IDEXX BioAnalytics case ID and IDEXX BioAnalytics sample ID; then slides were subjected to histopathologic analysis. Specimen and group information are outlined in **Table 1**.
Slide lettering represents the following tissues:
A= liver
B = adipose tissue

Microscopic changes in the liver were scored utilizing the Non-Alcoholic Fatty Liver Disease (NAFLD) scoring system for rodent models published (PLoS One. 2014; 9(12): e115922). Microscopic changes not covered by the NAFLD scoring system for rodent models and fibrosis were graded, as to severity, utilizing a standard grading system whereby 0 = no significant change, 1= minimal, 2 = mild, 3 = moderate, and 4 = severe. International Harmonization of Nomenclature and Diagnostic (INHAND) Criteria standards are used as the basis of evaluation-(https://www.toxpath.org/inhand.asp).

All samples were well preserved with minimal to undetected presence of autolytic artifacts which allowed for an adequate and accurate histological evaluation. The use of numerical grades provides a mechanism to calculate a total score lesion score which can be used to assess prevalence and severity of tissue changes within and between groups.

Results

Individual histopathology findings for each animal and organ are presented in **Table 2**. Summary of histopathology findings for each group are presented in **Table 3**. Annotated images are presented in **Appendix 1.**

In **Table 3**, the Mean Group Score for a tissue change is the mean of severity scores for all animals in the group, including animals for which a tissue change was not observed. The Mean Individual Score is the mean of severity scores only for animals with a scored tissue change in the Group. Mean Lesion Scores do not include animals for which a tissue change was not observed. No Significant Findings is the number of animals that did not have any significant findings for each organ. The Sum Score is the sum of all graded changes in each tissue for each animal.

A detailed summary and histological description evaluation of observed changes per organ are described below.

Liver

In all liver, hepatocellular hypertrophy, microvascular and macrovascular steatosis was detected. Inflammation, characterized by infiltrate of low to moderate number of mononuclear cells (histocytes, lymphocytes, plasma cells) and few granulocytic cells (neutrophils) was detected in seventeen of the eighteen animals. The microvascular steatosis was characterized by small clear vacuoles expanding the cytoplasm of the hepatocytes, without displacing the nuclei. The macrovascular steatosis was characterized by larger clear vacuoles expanding the cytoplasm of the hepatocytes displacing the nuclei. The hepatocellular hypertrophy was characterized by cellular enlargement of more than 1.5 times the normal hepatocytes diameter. The hepatocellular hypertrophy was the result of the microvesicular and macrovesicular steatosis. Areas of cell death/necrosis were not identified.

The NASH liver score was lesser in Group 2 – DR only (Mean Group Score of 7.7), followed by Group 3 – E4 only (Mean Group Score of 8.3), Group 5 – midpoint (Mean Group Score of 8.5) Group 4 – ED+DR (Mean Group Score of 8.7). Group 1 – Control had the highest Mean Group Score of 10.3. The lower NASH liver score in the treatment groups compared to the control group was interpreted as test-article related.

Fibrosis is best assessed with collage special stain such as Picrosirius red or Trichrome Masson. Fibrosis was assessed with H&E alone in this case. For more detailed fibrosis assessment, special stain could be done on the paraffin embedded tissue block. The fibrosis seen on H&E was minimal to mild and detected in all animals from the Control and DR groups, and in most animals from the E4 only (n=2), ED+DR (n=2), and Midpoint (n=5) groups. Fibrosis Mean Group Scores were comparable across all Groups.

A population of atypical round cells forming perivascular aggregates was detected in six of the eighteen animals. The atypical rounds cells were distinctively bordered with moderate amount of basophilic cytoplasm, large nuclei with clumped chromatin, moderate anisocytosis and anisokaryosis, and variable number of mitotic figures. These atypical round cells could represent an atypical hyperplastic lymphoid response or incipient/early lymphoma/round cell neoplasia. These atypical round cells infiltrate could be the result of a genetic manipulation depending on the transgenic background of these mice as it is not a typical finding in aged C57BL/6 mice. The atypical round cell infiltrates were present in the Control Group animals, therefore are considered background changes.

Inguinal adipose tissue

Minimal histiocytic infiltrate was detected in two animals from Group 3 – E4 only. The histiocytic was minimal and could represent incipient steatosis but could also be incidental and the result of minimal adipose tissue trauma. Infectious organisms were not detected. The clinical significance of this minimal histiocytic infiltrate is uncertain.

Minimal fibrosis was detected in one small area of the inguinal adipose tissue in one animal from Group 2 – DR only. This solitary area of minimal fibrosis was interpreted to be a background incidental finding.

Conclusion

1. Test article-related lower NASH liver score was identified in Group 2 – DR only, followed by Group 3 – E4 only, Group 5 – midpoint, and Group 4 – ED+DR when compared to Group 1 – Control. Fibrosis was identified in all Groups with similar frequency and severity. Special stain may help identifying more subtle difference in fibrosis across groups.
2. Atypical round cell infiltrates were observed in the liver of six of the eighteen mice and were interpreted as background findings (reactive lymphoid hyperplasia vs incipient lymphoma), possibly the result of the genetic

mutation of these mice.

3. Inguinal adipose tissue was normal in all but two animals from Group 3 – E4 only where minimal histiocytic infiltrate was identified and one animal from Group 2 – DR only where minimal fibrosis was detected. There was no evidence of steatosis. The changes detected in the adipose tissue of these three animals were minimal and likely not clinically/biologically relevant/significant.

Table 1. Group and Specimen information.

IBA Animal ID	Slide ID	Animal ID/Sample ID	Group ID	Tissue Depot
1	1B	A10-iWAT	Control	Inguinal white adiose tissue
2	2B	A11-iWAT	Control	Inguinal white adiose tissue
3	3B	A13-iWAT	Control	Inguinal white adiose tissue
1	1A	A10-Liver	Control	Liver
2	2A	A11-Liver	Control	Liver
3	3A	A13-Liver	Control	Liver
4	4B	A2-iWAT	DR Only	Inguinal white adiose tissue
5	5B	A3-iWAT	DR Only	Inguinal white adiose tissue
6	6B	A4-iWAT	DR Only	Inguinal white adiose tissue
4	4A	A2-Liver	DR Only	Liver
5	5A	A3-Liver	DR Only	Liver
6	6A	A4-Liver	DR Only	Liver
7	7B	1167-iWAT	E4 Only	Inguinal white adiose tissue
8	8B	1192-iWAT	E4 Only	Inguinal white adiose tissue
9	9B	1193-iWAT	E4 Only	Inguinal white adiose tissue
7	7A	1167-Liver	E4 Only	Liver
8	8A	1192-Liver	E4 Only	Liver
9	9A	1193-Liver	E4 Only	Liver
10	10B	1117-iWAT	E4+DR	Inguinal white adiose tissue
11	11B	1157-iWAT	E4+DR	Inguinal white adiose tissue
12	12B	1206-iWAT	E4+DR	Inguinal white adiose tissue
10	10A	1117-Liver	E4+DR	Liver
11	11A	1157-Liver	E4+DR	Liver
12	12A	1206-Liver	E4+DR	Liver
13	13B	1234-iWAT	Midpoint	Inguinal white adiose tissue
14	14B	1242-iWAT	Midpoint	Inguinal white adiose tissue
15	15B	1243-iWAT	Midpoint	Inguinal white adiose tissue
13	13A	1234-Liver	Midpoint	Liver
14	14A	1242-Liver	Midpoint	Liver
15	15A	1243-Liver	Midpoint	Liver
16	16B	A29-iWAT	Midpoint	Inguinal white adiose tissue
17	17B	A30-iWAT	Midpoint	Inguinal white adiose tissue
18	18B	A39-iWAT	Midpoint	Inguinal white adiose tissue
16	16A	A29-Liver	Midpoint	Liver
17	17A	A30-Liver	Midpoint	Liver
18	18A	A39-Liver	Midpoint	Liver

Table 2: Individual Animal Histopathology Findings

Group 1 - Control # Organs	2					
Organ Number- Tissue	**1**	**2**	**3**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
1- Inguinal adipose tissue						
Fibrosis	0	0	0	0	0	
Infiltrate, mononuclear	0	0	0	0	0	
Artifact	0	0	0	0	0	
Sum - Scores:	0	0	0		0	
No Significant Findings:	3					

Organ Number- Tissue	**1**	**2**	**3**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
2- Liver - NASH						
Steatosis, macrovascular	3	3	2	3	2.7	
Steatosis, microvascular	2	2	3	3	2.3	
Hypertrophy	3	3	3	3	3	
Inflammation	3	2	2	3	2.3	
Artifact	0	0	0	0	0	
Sum - Scores:	11	10	10		10.3	
No Significant Findings:	0					

Organ Number- Tissue	**1**	**2**	**3**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
3- Liver - other						
Fibrosis	2	1	2	3	1.7	
Infiltrate, atypical round cell	2	0	0	1	0.7	
Artifact	0	0	0	0	0	
Sum - Scores:	4	1	2		2.3	
No Significant Findings:	0					

Table 2: Individual Animal Histopathology Findings

Group 2 - DR Only # Organs	2					
Organ Number- Tissue	**4**	**5**	**6**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
1- Inguinal adipose tissue						
Fibrosis	1	0	0	1	0.3	
Infiltrate, mononuclear	0	0	0	0	0.0	
Artifact	0	0	0	0	0.0	
Sum - Scores:	1	0	0		0.3	
No Significant Findings:	2					

Organ Number- Tissue	**4**	**5**	**6**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
2- Liver - NASH						
Steatosis, macrovascular	1	1	1	3	1	
Steatosis, microvascular	1	3	3	3	2.3	
Hypertrophy	2	3	2	3	2.3	
Inflammation	3	1	2	3	2.0	
Artifact	0	0	0	0	0.0	
Sum - Scores:	7	8	8		7.7	
No Significant Findings:	0					

Organ Number- Tissue	**4**	**5**	**6**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
3- Liver - other						
Fibrosis	2	2	1	3	1.7	
Infiltrate, atypical round cell	0	1	1	2	0.7	
Artifact	0	0	0	0	0.0	
Sum - Scores:	2	3	2		2.3	
No Significant Findings:	0					

Table 2: Individual Animal Histopathology Findings

Group 3 - E4 Only # Organs 24						
Organ Number- Tissue	**7**	**8**	**9**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
1- Inguinal adipose tissue						
Fibrosis	0	0	0	0	0	
Infiltrate, mononuclear	1	1	0	2	0.7	
Artifact	0	0	0	0	0.0	
Sum - Scores:	1	1	0		0.7	
No Significant Findings:	1					

Organ Number- Tissue	**7**	**8**	**9**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
2- Liver - NASH						
Steatosis, macrovascular	2	2	2	3	2	
Steatosis, microvascular	3	2	1	3	2.0	
Hypertrophy	3	3	2	3	2.7	
Inflammation	2	2	1	3	1.7	
Artifact	0	0	0	0	0	
Sum - Scores:	10	9	6		8.3	
No Significant Findings:	0					

Organ Number- Tissue	**7**	**8**	**9**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
3- Liver - other						
Fibrosis	2	1	0	2	1.0	
Infiltrate, atypical round cell	2	1	0	2	1.0	
Artifact	0	0	0	0	0.0	
Sum - Scores:	4	2	0		2.0	
No Significant Findings:	1					

Table 2: Individual Animal Histopathology Findings

Group 4 - ED+DR # Organs	2					
Organ Number- Tissue	**10**	**11**	**12**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
1- Inguinal adipose tissue						
Fibrosis	0	0	0	0	0	
Infiltrate, mononuclear	0	0	0	0	0	
Artifact	0	0	0	0	0	
Sum - Scores:	0	0	0		0	
No Significant Findings:	3					

Organ Number- Tissue	**10**	**11**	**12**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
2- Liver - NASH						
Steatosis, macrovascular	1	1	2	3	1.3	
Steatosis, microvascular	3	2	3	3	2.7	
Hypertrophy	3	2	3	3	2.7	
Inflammation	2	3	1	3	2.0	
Artifact	0	0	0	0	0.0	
Sum - Scores:	9	8	9		8.7	
No Significant Findings:	0					

Organ Number- Tissue	**10**	**11**	**12**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**3**					
3- Liver - other						
Fibrosis	0	2	1	2	1.0	
Infiltrate, atypical round cell	0	0	1	1	0.3	
Artifact	0	0	0	0	0.0	
Sum - Scores:	0	2	2		1.3	
No Significant Findings:	1					

Table 2: Individual Animal Histopathology Findings

Group 5 - Midpoint # Organs 24									
Organ Number- Tissue	**13**	**14**	**15**	**16**	**17**	**18**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**6**								
1- Inguinal adipose tissue									
Fibrosis	0	0	0	0	0	0	0	0	
Infiltrate, mononuclear	0	0	0	0	0	0	0	0	
Artifact	0	0	0	0	0	0	0	0	
Sum - Scores:	0	0	0	0	0	0		0	
No Significant Findings:	6								

Organ Number- Tissue	**13**	**14**	**15**	**16**	**17**	**18**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**6**								
2- Liver - NASH									
Steatosis, macrovascular	1	2	2	3	2	2	6	2	
Steatosis, microvascular	2	3	2	2	3	3	6	2.5	
Hypertrophy	2	3	2	3	3	3	6	2.7	
Inflammation	1	1	2	1	2	1	6	1.3	
Artifact	0	0	0	0	0	0	0	0.0	
Sum - Scores:	6	9	8	9	10	9		8.5	
No Significant Findings:	0								

Organ Number- Tissue	**13**	**14**	**15**	**16**	**17**	**18**	**# Abnormal**	**Mean Group Score**	**Notes:**
n=	**6**								
3- Liver - other									
Fibrosis	0	2	1	2	2	1	5	1.3	
Infiltrate, atypical round cell	0	0	0	0	0	0	0	0.0	
Artifact	0	0	0	0	0	0	0	0.0	
Sum - Scores:	0	2	1	2	2	1		1.3	
No Significant Findings:	1								

Table 3: Summary of Histopathological Findings

Organ Number- Tissue	Group 1 - Control # Abnormal	Group 1 - Control Mean Group Score	Group 1 - Control Mean Lesion Score	Group 2 - DR Only # Abnormal	Group 2 - DR Only Mean Group Score	Group 2 - DR Only Mean Lesion Score
	n= 3			n= 3		
1- Inguinal adipose tissue						
Fibrosis	0	0.0		1	0.3	1.0
Infiltrate, mononuclear	0	0.0		0	0.0	
Artifact	0	0.0		0	0.0	
Sum - Scores:	0	0.0	0.0	1	0.3	1.0
No Significant Findings:	3			2		
	n= 3			n= 3		
2- Liver - NASH						
Steatosis, macrovascular	3	2.7	2.7	3	1.0	1.0
Steatosis, microvascular	3	2.3	2.3	3	2.3	2.3
Hypertrophy	3	3.0	3.0	3	2.3	2.3
Inflammation	3	2.3	2.3	3	2.0	2.0
Artifact	0	0.0		0	0.0	
Sum - Scores:	12	10.3	10.3	12	7.7	7.7
No Significant Findings:	0			0		
	n= 3			n= 3		
3- Liver - other						
Fibrosis	3	1.7	1.7	3	1.7	1.7
Infiltrate, atypical round cell	1	0.7	2.0	2	0.7	1.0
Artifact	0	0.0		0	0.0	
Sum - Scores:	4	2.3	3.7	5	2.3	2.7
No Significant Findings:	0			0		

Table 3: Summary of Histopathological Findings

Organ Number- Tissue	Group 3 - E4 Only # Abnormal	Group 3 - E4 Only Mean Group Score	Group 3 - E4 Only Mean Lesion Score	Group 4 - ED+DR # Abnormal	Group 4 - ED+DR Mean Group Score	Group 4 - ED+DR Mean Lesion Score	Group 5 - Midpoint # Abnormal	Group 5 - Midpoint Mean Group Score	Group 5 - Midpoint Mean Lesion Score
	n= 3			n= 3			n= 6		
1- Inguinal adipose tissue									
Fibrosis	0	0.0		0	0.0		0	0.0	
Infiltrate, mononuclear	2	0.7	1.0	0	0.0		0	0.0	
Artifact	0	0.0		0	0.0		0	0.0	
Sum - Scores:	2	0.7	1.0	0	0.0	0.0	0	0.0	0.0
No Significant Findings:	1			3			6		
	n= 3			n= 3			n= 6		
2- Liver - NASH									
Steatosis, macrovascular	3	2.0	2.0	3	1.3	1.3	6	2.0	2.0
Steatosis, microvascular	3	2.0	2.0	3	2.7	2.7	6	2.5	2.5
Hypertrophy	3	2.7	2.7	3	2.7	2.7	6	2.7	2.7
Inflammation	3	1.7	1.7	3	2.0	2.0	6	1.3	1.3
Artifact	0	0.0		0	0.0		0	0.0	
Sum - Scores:	12	8.3	8.3	12	8.7	8.7	24	8.5	8.5
No Significant Findings:	0			0			0		
	n= 3			n= 3			n= 6		
3- Liver - other									
Fibrosis	2	1.0	1.5	2	1.0	1.5	5	1.3	1.6
Infiltrate, atypical round cell	2	1.0	1.5	1	0.3	1.0	0	0.0	
Artifact	0	0.0		0	0.0		0	0.0	
Sum - Scores:	4	2.0	3.0	3	1.3	2.5	5	1.3	1.6
No Significant Findings:	1			1			1		

Appendix 1
Annotated Images

Company: Texas Tech University
Study Director: Vijay Hegde
Study ID: E4orf1 as a novel anti-NAFLD agent
IDEXX BioAnalytics Case ID: 103046-2024

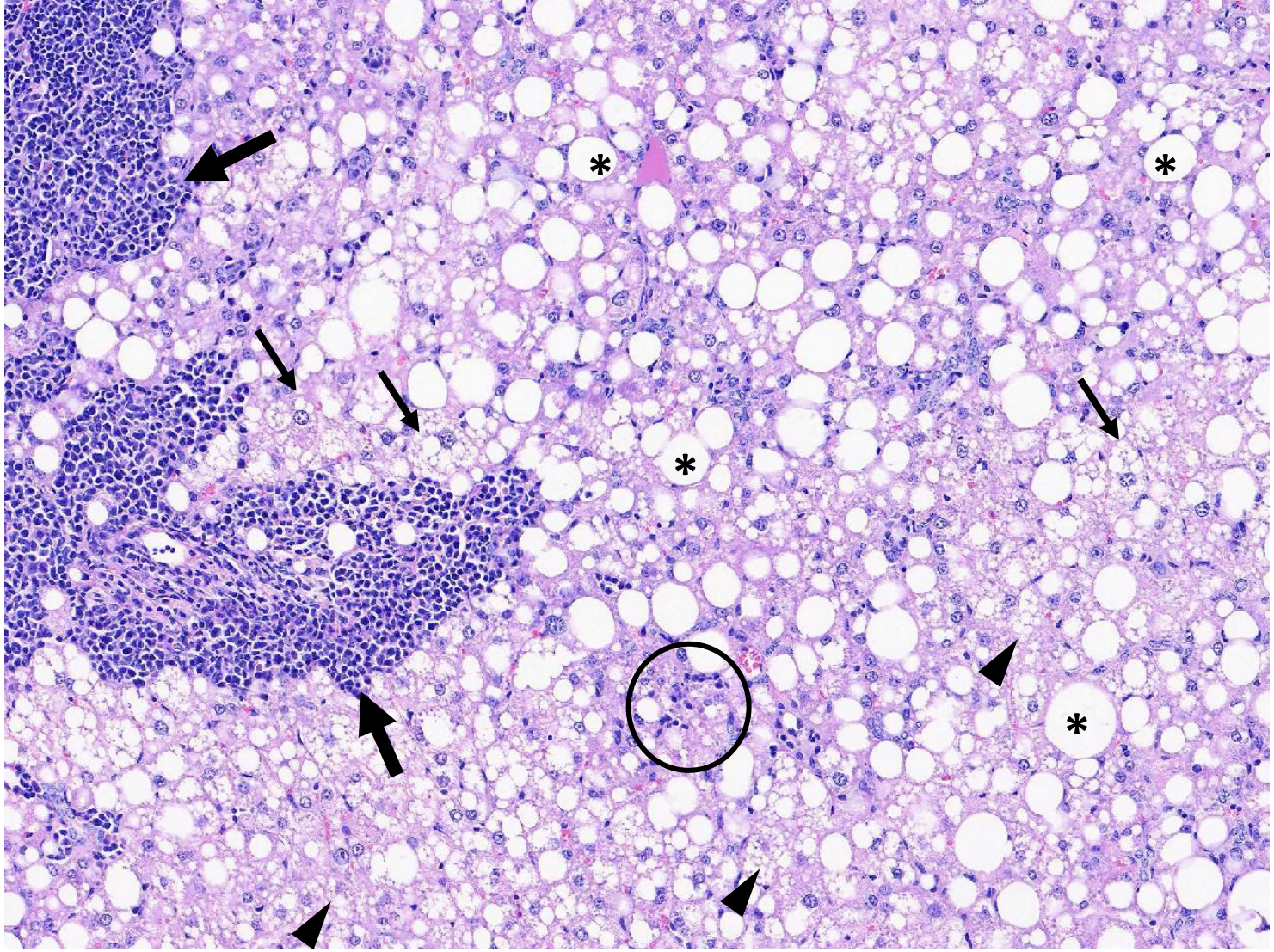

Figure 1. IBA sample ID 1, Texas Tech ID 1A, Control Group, C57BL/6 mouse, 20 X magnification, Hematoxylin and Eosin, liver. Macrovesicular steatosis (asterisk), microvesicular steatosis (arrowhead), hypertrophy (arrow), inflammation (circle), atypical round cell infiltrate (bold arrow). Fibrosis is difficult to visualize without special stain. Fibrosis was not evenly distributed in the liver and was not readily visible in this image.

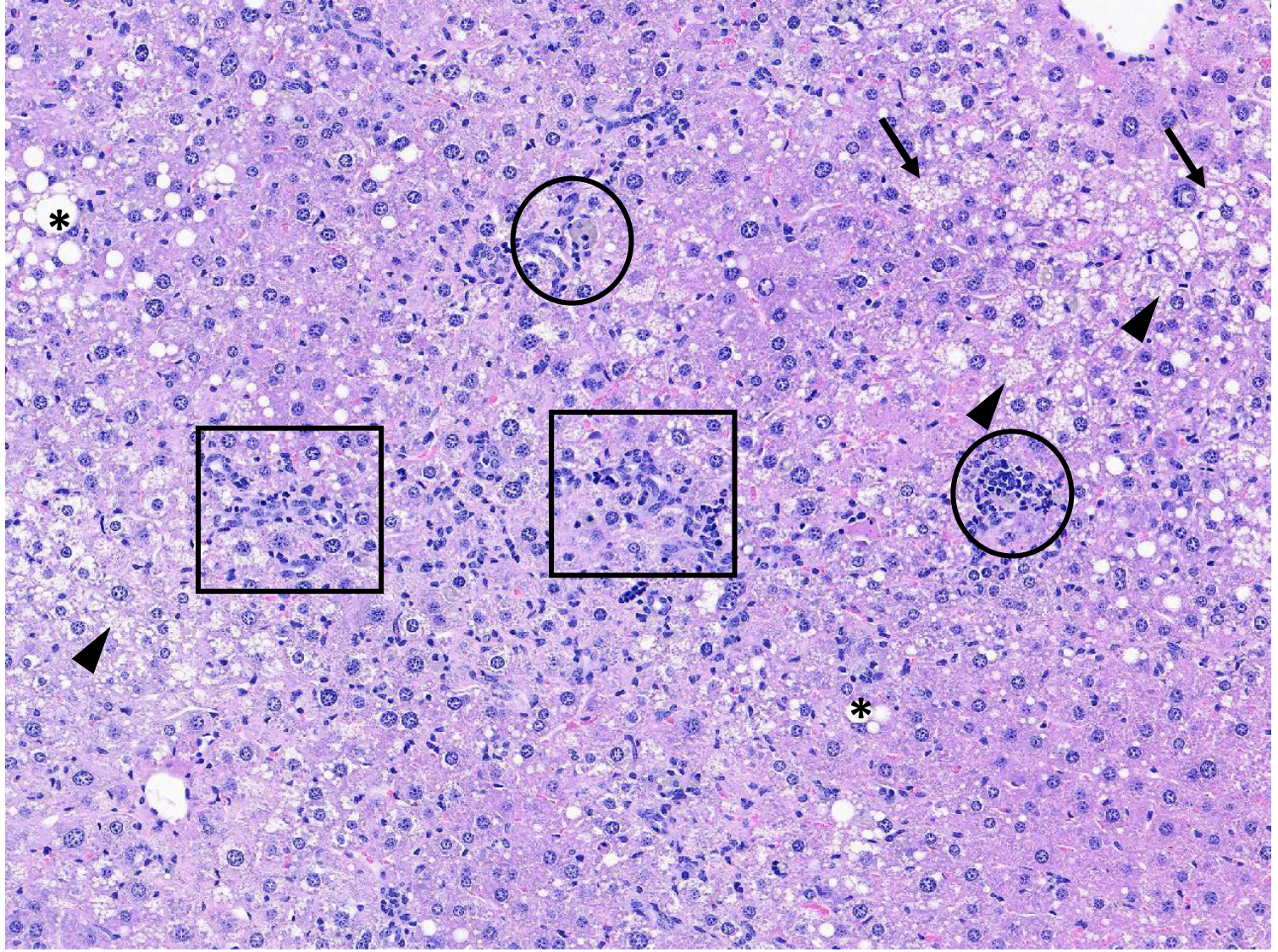

Figure 2. IBA sample ID 4, Texas Tech ID 4A, DR Group, C57BL/6 mouse, 20 X magnification, Hematoxylin and Eosin, liver. Macrovesicular steatosis (asterisk), microvesicular steatosis (arrowhead), hypertrophy (arrow), inflammation (circle), fibrosis (rectangle). Fibrosis is difficult to visualize without special stain.

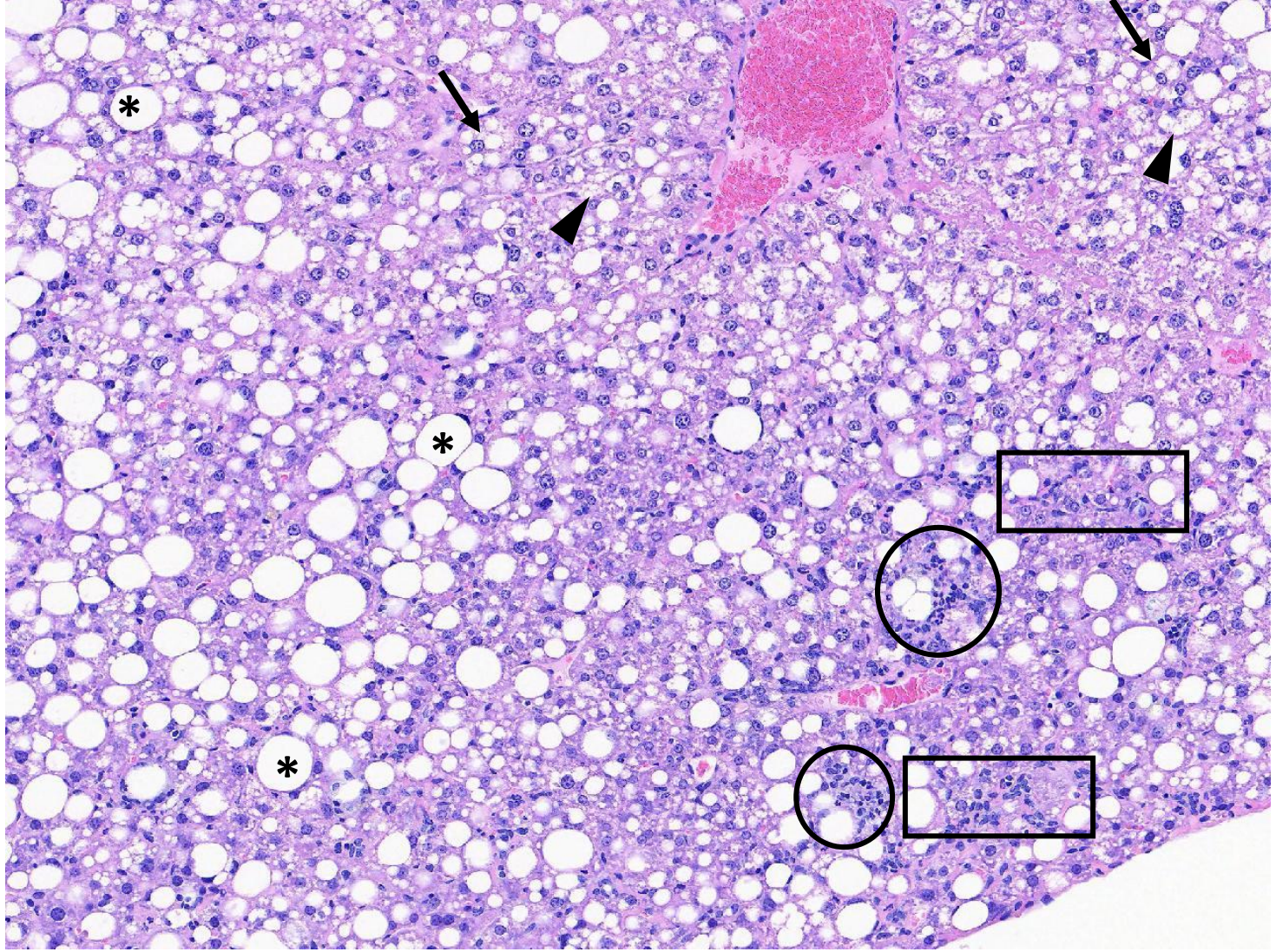

Figure 3. IBA sample ID 8, Texas Tech ID 8A, E4 only Group, C57BL/6 mouse, 20 X magnification, Hematoxylin and Eosin, liver. Macrovesicular steatosis (asterisk), microvesicular steatosis (arrowhead), hypertrophy (arrow), inflammation (circle), fibrosis (rectangle). Fibrosis is difficult to visualize without special stain.

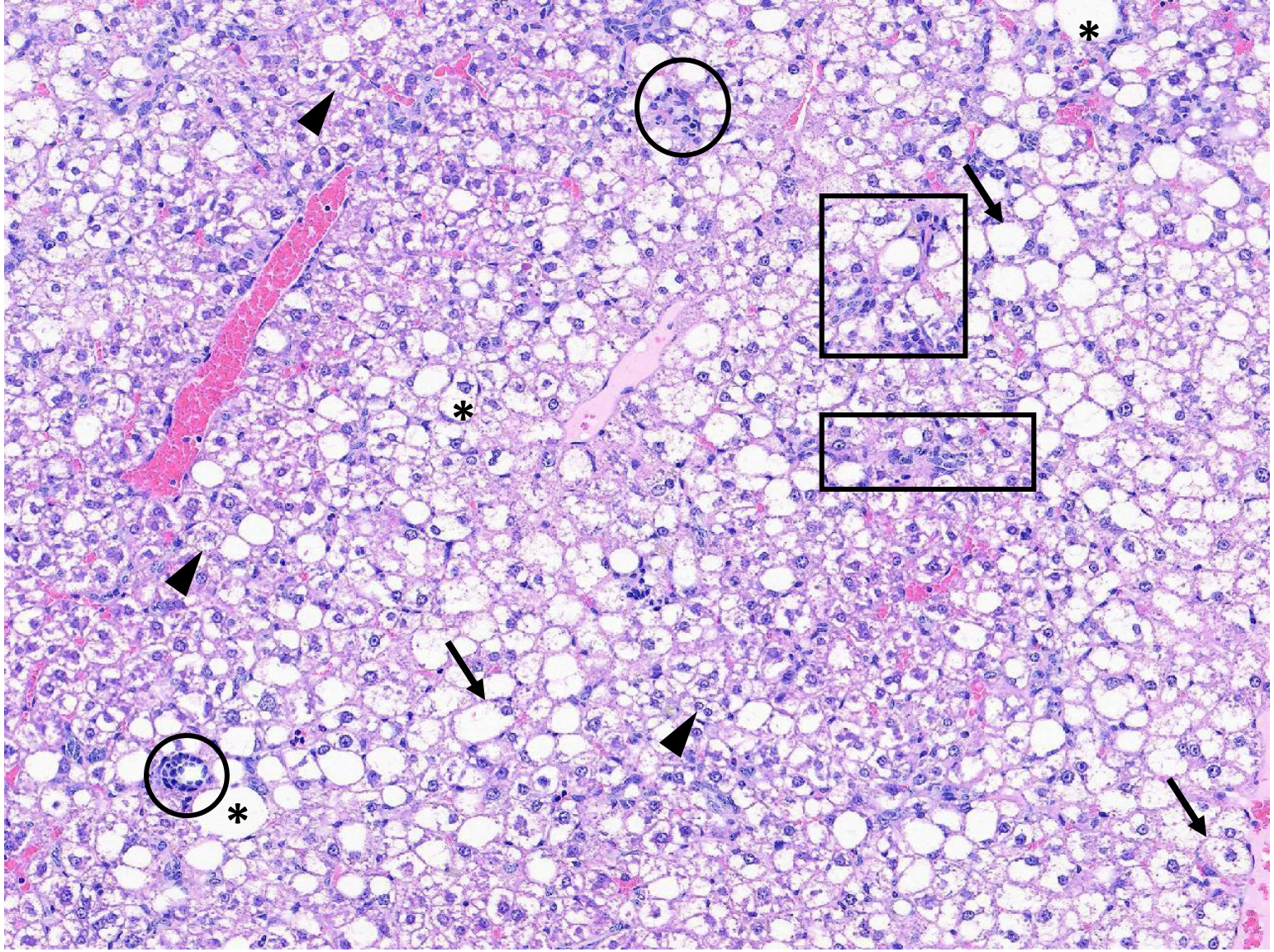

Figure 4. IBA sample ID 12, Texas Tech ID 12A, E4+DR Group, C57BL/6 mouse, 20 X magnification, Hematoxylin and Eosin, liver. Macrovesicular steatosis (asterisk), microvesicular steatosis (arrowhead), hypertrophy (arrow), inflammation (circle), fibrosis (rectangle). Fibrosis is difficult to visualize without special stain.

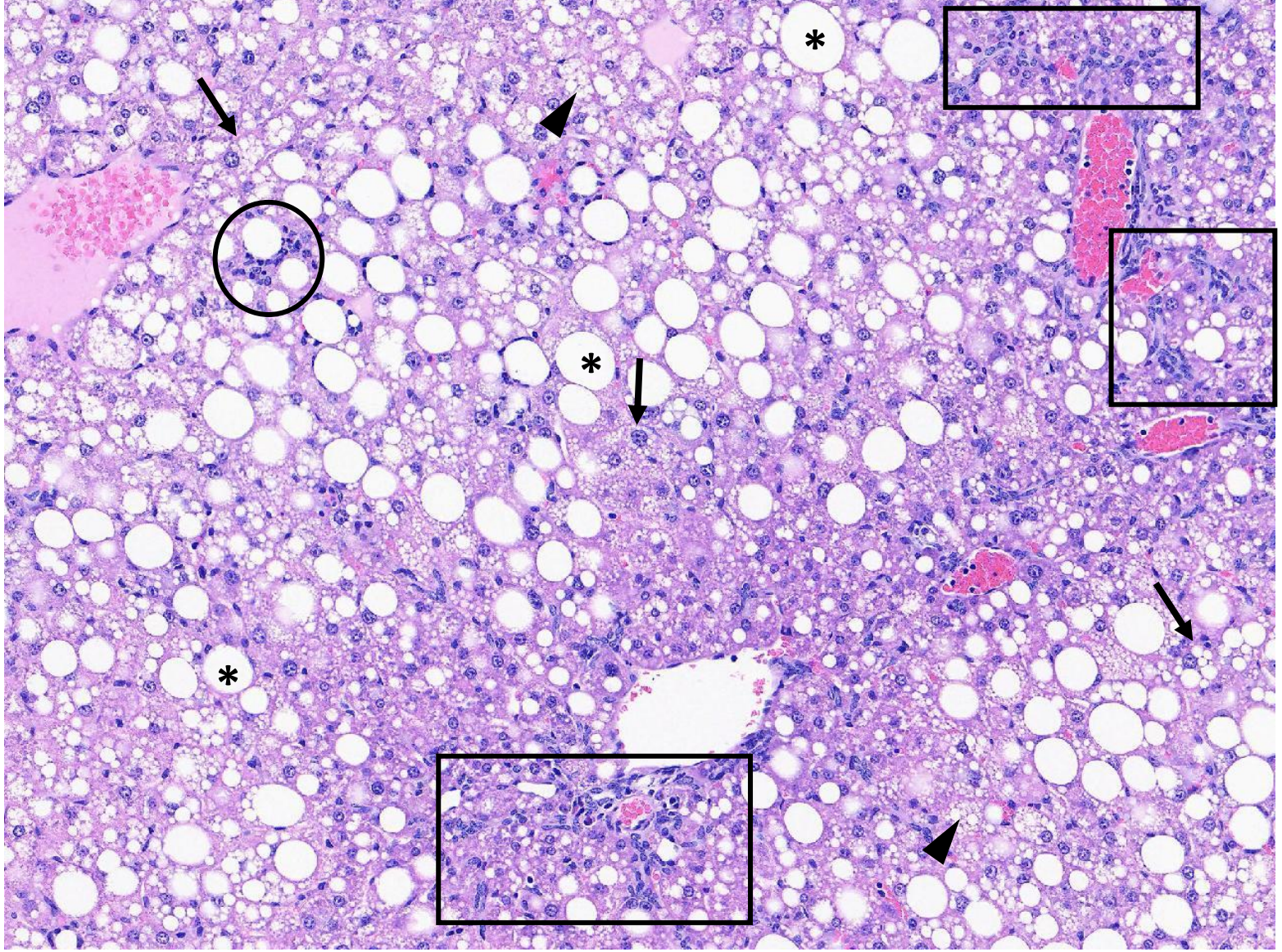

Figure 5. IBA sample ID 14, Texas Tech ID 14A, MidPoint Group, C57BL/6 mouse, 20 X magnification, Hematoxylin and Eosin, liver. Macrovesicular steatosis (asterisk), microvesicular steatosis (arrowhead), hypertrophy (arrow), inflammation (circle), fibrosis (rectangle). Fibrosis is difficult to visualize without special stain.