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Supplementary information

Sex differences orchestrated by androgens at single-cell resolution

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Supplementary Information for

Sex differences orchestrated by androgens at single-cell resolution

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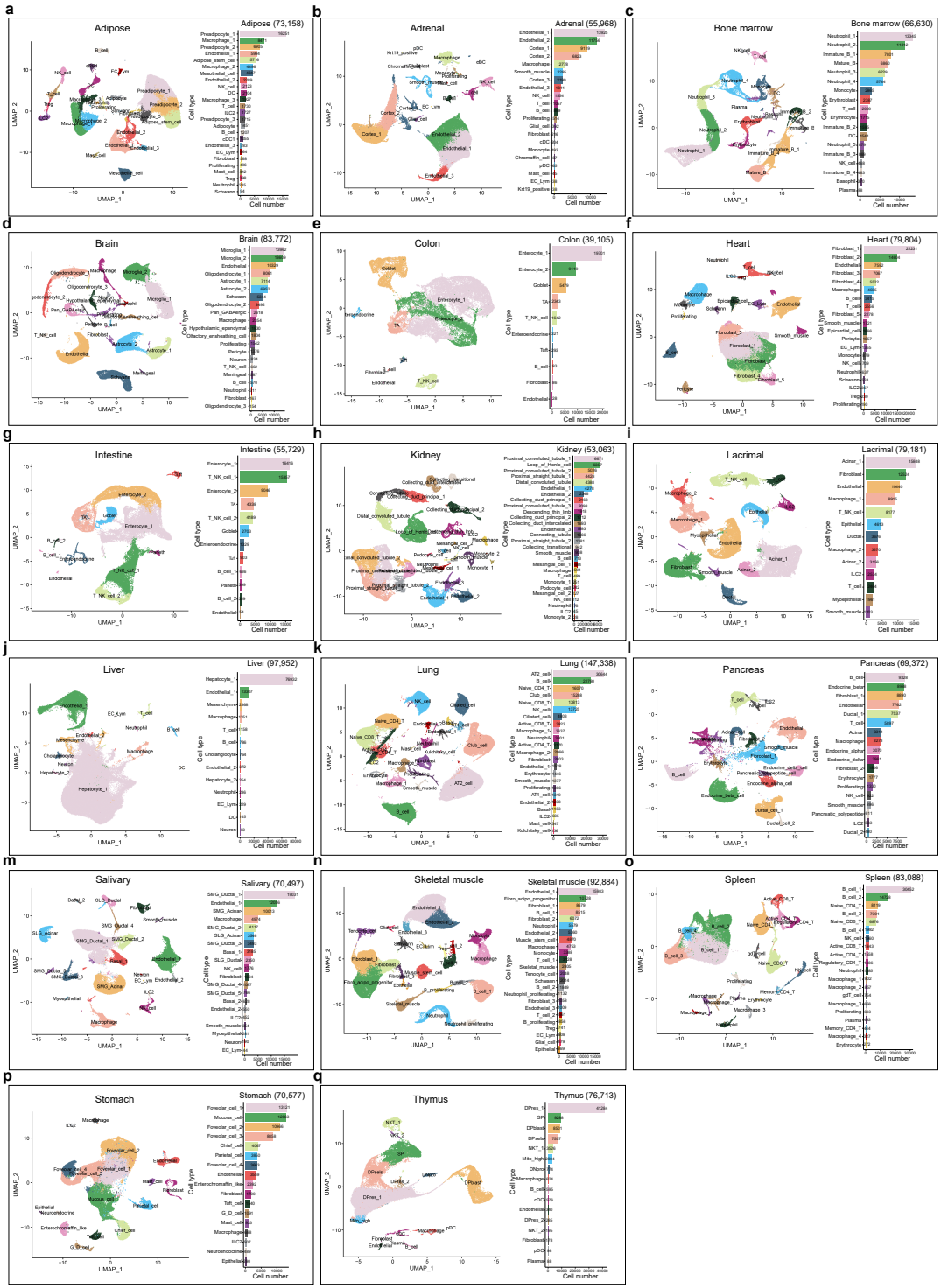
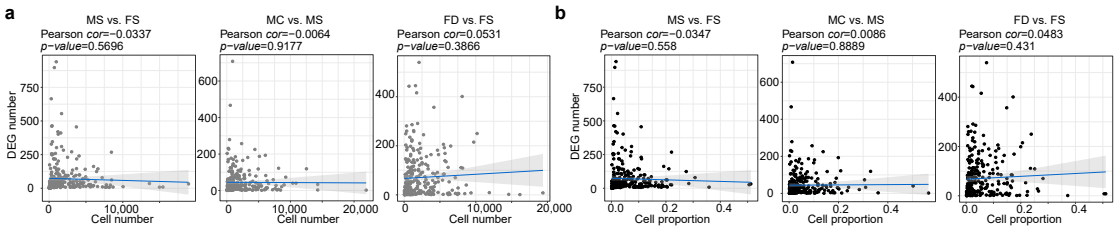


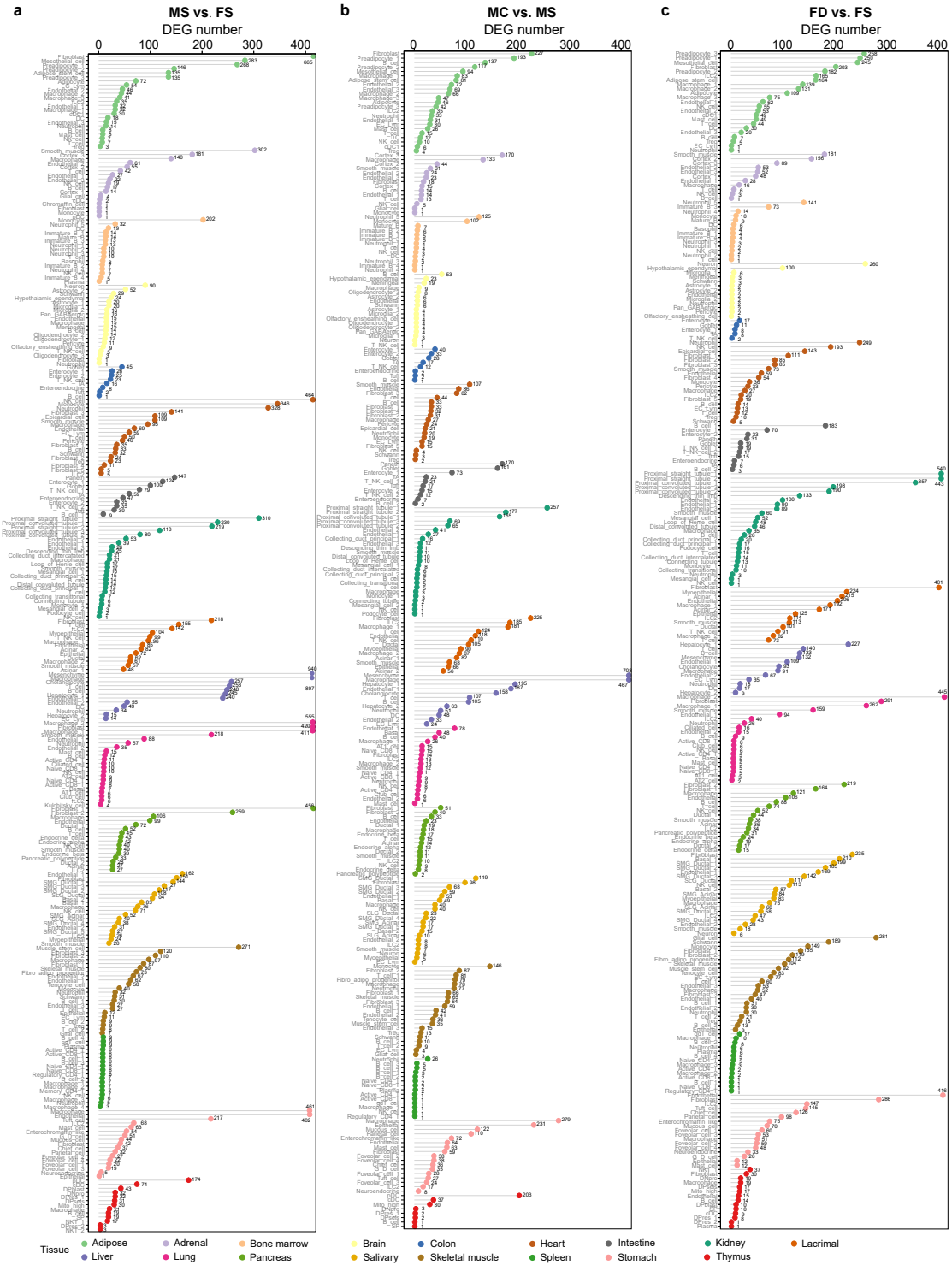
Supplementary Fig. 1 | Cell types across tissues.

a-q, UMAP plots showing the cellular compartments for the adipose (**a**), adrenal (**b**), bone marrow (**c**), brain (**d**), colon (**e**), heart (**f**), intestine (**g**), kidney (**h**), lacrimal gland (**i**), liver (**j**), lung (**k**), pancreas (**l**), salivary gland (**m**), skeletal muscle (**n**), spleen (**o**), stomach (**p**) and thymus (**q**) tissue of MS, MC, FS and FD mice.



Supplementary Fig. 2 | The relationship between DEG numbers and cell numbers or DEG numbers and cell proportions.

a, Scatter plot showing the correlation between DEG numbers and cell numbers across cell types in three comparisons of MS vs. FS, MC vs. MS and FD vs. FS. **b**, Scatter plot showing the correlation between DEG numbers and cell proportions across cell types in three comparisons of MS vs. FS, MC vs. MS and FD vs. FS. In **a** and **b**, *P* values are shown; the test statistic is based on Pearson's product moment correlation coefficient and follows a t distribution; level of confidence interval is 0.95.



Supplementary Fig. 3 | Identification of the DEGs across all cell types.

a-c, Lollipop plot showing cell types ranked by the number of DEGs in each tissue based on the three comparisons of MS vs. FS (**a**), MC vs. MS (**b**) and FD vs. FS (**c**). The upper x-axis limit was set to 400.

Supplementary Methods

Dissociation of tissues into single-cell suspensions

1. Adipose

Adipose tissue was dissociated using an Adipose Tissue Dissociation Kit (Miltenyi, 130-105-808) with a modified protocol. Abdominal adipose tissue that weighed approximately 0.5 g-1.0 g was collected and cut into small pieces of 2–4 mm after connective tissue removal (especially the attached epididymis for male mice and the uterus for female mice). Tissue pieces were then transferred into a gentleMACS™ C Tube (Miltenyi, 130-093-237) containing the enzyme mixture (4.7 mL of DMEM, 200 μL of Enzyme D, 100 μL of Enzyme R, and 25 μL of Enzyme A) and dissociated into single cells with Dissociator using the 37C_mr_ATDK_1 program. Cells were collected by a short centrifugation step at 300 g. The cell suspension was gently mixed using a pipette and then applied to a 100 μm cell strainer placed in a 15 mL tube. Ten milliliters of DMEM supplemented with 10% FBS (DMEM-FBS) was added to a gentleMACS™ C tube and then transferred to a 100 μm cell strainer to collect the remaining cells. Cells were collected by a centrifugation step at 400 × g for 10 minutes at 4 °C. After removal of the supernatant, the cells were resuspended in 1 mL of red blood cell lysis buffer (Miltenyi, 130-094-183) and incubated for 2 minutes. Immediately, the cell suspension was applied to a70-μm cell strainer placed in a new 15 mL tube to remove lipid droplets as well as cell clumps. The cell strainer was washed with 8 mL of PBS. Cells were collected by a centrifugation step at 500 × g for 5 minutes and resuspended in 50 μL of 0.04% BSA in DPBS.

2. Adrenal gland

The adrenal tissue was dissociated and transferred into a 1.5 mL tube containing the enzyme mixture (500 μL of collagenase II supplemented with 0.05 μL of Y-27632) after the removal of connective tissues. The adrenal tissue was cut into four small pieces and placed in a thermoshaker for an incubation step at 1700 rpm for 10 minutes at 37 °C. During incubation, the adrenal suspension was pipetted 30 times every 5 minutes to

completely disrupt the cell clumps. The cell pellet was resuspended in 500 µL of TrypLE and collected by centrifugation at 1700 rpm for 3 minutes. The cell pellet was resuspended in 500 µL of TrypLE and incubated in a thermoshaker at 1700 rpm for 9 minutes at 37 °C. The cell suspension was pipetted 20 times every 3 minutes to completely disrupt the cell clumps. The digestion reaction was quenched by adding 1 mL of DMEM supplemented with 10% FBS, and the cell suspension was applied to a 70-µm cell strainer placed in a new 15 mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. Centrifugation at 1700 rpm for 3 minutes was performed to collect cells. The cells were resuspended in 1 mL of red blood cell lysis buffer and incubated for 2 minutes at room temperature, after which 4 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 500 µL of 0.04% BSA in DPBS.

3. Bone marrow

All muscle and connective tissues were removed from the left tibia and left femur. The joints were cut off, and the bone marrow was pushed out into a 15 mL tube with a syringe using 1 mL of cold PBS for a total of 5 times. Bone marrow was next collected by centrifugation at 400 × g for 5 minutes and then resuspended in 1 mL of collagenase/hyaluronidase (STEMCELL, 07912). The bone marrow suspension was transferred into a 1.5 mL tube and placed in a thermoshaker for an incubation step at 1500 rpm for 20 minutes at 37 °C. During incubation, the bone marrow suspension was pipetted 30 times every 5 minutes to completely disrupt the cell clumps. The cell suspension was transferred to a 15 mL tube, and the digestion reaction was quenched by adding 5 mL of DMEM supplemented with 10% FBS. The cell suspension was applied to a 70-µm cell strainer placed in a new 15 mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. Centrifugation at 300 × g for 5 minutes was performed to collect the cells. The cells were resuspended in 2 mL of red blood cell lysis buffer (Miltenyi, 130-094-183) and incubated for 2 minutes, after which 8 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 1 mL of PBS. The cell suspension was further

diluted one to ten with 0.04% BSA in DPBS.

4. Brain

The brains were dissociated using a Brain Dissociation Kit (Miltenyi, 130-107-677) with a modified protocol. After olfactory bulb removal, the brain was cut into approximately 0.5 cm pieces in a 10 cm dish with cold DPBS. Tissue pieces were then transferred into a gentleMACS™ C tube containing enzyme mixture 1 (50 μL of Enzyme R, 1900 μL of Buffer Z), and then enzyme mixture 2 (20 μL of Buffer Y, 10 μL of Enzyme A) was transferred to the C tube. The C tube was placed in Dissociator with the 37C_ABDK_01 program. After incubation, the cells were briefly centrifuged at 300 × g for 10 seconds. The cells were resuspended in 8 mL of cold DPBS and then applied to a 70 μm cell strainer placed in a 15-mL tube. The C tube was washed with 2 mL of DPBS, which was also applied to the cell strainer. The cells were collected by a centrifugation step at 300 × g for 10 minutes at 4 °C and carefully resuspended in 3100 μL of cold DPBS, followed by gentle addition of 900 μL of Debris Removal Solution. The cell suspension was gently mixed using a pipette and overlaid with 4 mL of cold DPBS. Centrifugation at 3000 × g for 10 minutes at 4 °C was further performed, resulting in the formation of three phases, among which the bottom phase was preserved. Cells were resuspended in 14 mL of cold DPBS, followed by gentle inversion 3 times. Cells were collected by a centrifugation step at 1000 × g for 10 minutes at 4 °C and resuspended in 1 mL of red blood cell lysis buffer (Miltenyi, 130-094-183) for a 10-minute incubation on ice. Ten milliliters of cold PB buffer were added, and the cells were centrifuged at 300 × g for 10 minutes at 4 °C. Cells were further resuspended in 500 μL of 0.04% BSA in DPBS.

5. Colon

The colon was opened longitudinally, flushed with ice-cold PBS and cut into approximately 4 mm-long pieces. Then, the pieces were incubated in 5 mM EDTA at 37 °C for 10 minutes. After settling naturally, the EDTA was discarded, whole-epithelial segments were isolated by vigorous pipetting in 5 ml of ice-cold PBS 10 times, and this

step was repeated 3 times. A centrifugation step at 1500 rpm for 3 minutes was performed to collect the cells. Three milliliters of TrypLE supplemented with DNase I and Y-27632 was added at 37 °C for 20 minutes, and the samples were washed once with cold PBS. Then, 1 mL of fresh TrypLE supplemented with DNase I and Y-27632 was added to digest segments into single cells at 37 °C for 30-50 minutes. Fresh TrypLE supplemented with DNase I and Y-27632 was added every 20 minutes. The cells were filtered through a 70-μm cell strainer after termination of digestion and then resuspended in 3 mL of red blood cell lysis buffer for a 1-minute incubation at room temperature. Cells were centrifuged at 300 × g for 5 minutes and resuspended in 200 μL of 0.04% BSA in DPBS.

6. Heart

The heart was dissociated using a neonatal heart dissociation kit (Miltenyi, 130-098-373) with a modified protocol. The heart was harvested and transferred to a 10-cm dish containing cold DPBS, followed by the removal of all connective tissues. The heart was cut into 2-3-mm pieces and washed in DPBS to remove the remaining blood. The enzyme mixture was generated by adding 2362.5 μL of enzyme mixture 1 (62.5 μL of Enzyme P, 2300 μL of Buffer X) to 137.5 μL of enzyme mixture 2 (25 μL of Buffer Y, 12.5 μL of Enzyme A and 100 μL of Enzyme D). The tissue pieces were transferred into a gentleMACS™ C tube, and 2.5 mL of enzyme mix was added. Tissue pieces were then dissociated into single cells with Dissociator using the 37C_mr_NHDK_1 program. After dissociation, the cells were briefly centrifuged at 300 × g for 10 seconds at 4 °C, and 7.5 mL of DMEM-FBS was added to quench the enzymatic reaction. The cell suspension was then applied to a 70-μm cell strainer placed in a 50 mL tube, and then, the C tube was washed with 3 mL of DMEM-FBS, which was also applied to the cell strainer, followed by a centrifugation step at 600 × g for 5 minutes at 4 °C. Cells were resuspended in 1 mL of PEB buffer and then transferred into a 15 mL tube. Red blood cells were lysed via incubation in 10 mL of red blood cell lysis buffer (Miltenyi, 130-094-183) for 2 minutes. Cells were centrifuged at 600 × g for 5 minutes and resuspended in 10 mL of DPBS supplemented with 15 μL of Enzyme A, followed by

centrifugation at 500 × g for 5 minutes. Cells were resuspended in 10 mL of DPBS and then applied to a 40 μm cell strainer placed in a 15-mL tube. Cells were collected by centrifugation at 500 × g for 5 minutes and resuspended in 50 μL of 0.04% BSA in DPBS.

7. Intestine

The small intestine was opened longitudinally and cut into approximately 2-mm-long pieces. Then, the pieces were incubated in 2 mM EDTA on ice for 30 minutes. After settling naturally, the EDTA was discarded, whole-epithelial segments were isolated by vigorous pipetting in 5 mL of ice-cold PBS 10 times, and this step was repeated 3 times. A centrifugation step at 1500 rpm for 3 minutes was performed to collect the cells. Three milliliters of trypsin supplemented with DNase I and Y-27632 was added to digest segments into single cells at 37 °C for 20-30 minutes, and the cells were filtered through a 70 μm cell strainer after termination of digestion and then resuspended in 3 mL of red blood cell lysis buffer for 1 minute of incubation at room temperature. Cells were centrifuged at 300 × g for 5 minutes and resuspended in 0.04% BSA in DPBS.

8. Kidney

The kidney was dissociated and transferred into a 1.5 mL tube containing the enzyme mixture (1 mL of DMEM with 2% FBS, 40 μL of liberase, 1 μL of DNase I) after connective tissue removal (especially the attached capsule on the surface of the kidney). The kidney was cut into small pieces and placed on a thermoshaker for an incubation step at 1500 rpm for 30 minutes at 37 °C. During incubation, the kidney suspension was shaken 60 times every 10 minutes to completely disrupt cell clumps. The cells were resuspended in 500 μL of TrypLE and collected by centrifugation at 1700 rpm for 3 minutes. Kidney cells were resuspended in 1 mL of TrypLE and incubated in a thermoshaker at 1500 rpm for 20 minutes at 37 °C. The cell suspension was pipetted 20 times every 5 minutes to completely disrupt the cell clumps. The digestion reaction was quenched by adding 1 mL of DMEM supplemented with 10% FBS, and the cell suspension was applied to a 70 μm cell strainer placed in a new 15 mL tube. The cell

strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. A centrifugation step at 1700 rpm for 3 minutes was performed to collect cells. The cells were resuspended in 1 mL of red blood cell lysis buffer and incubated for 2 minutes at room temperature, after which 4 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 2 mL of 0.04% BSA in DPBS.

9. Lacrimal gland

The lacrimal tissue was dissociated and transferred to a 5 mL tube containing the enzyme mixture (5 mL of collagenase II, 0.5 μL of Y-27632) after connective tissue removal. The extraorbital lacrimal gland was cut into small pieces and placed on a thermoshaker for a rotary incubation step at 15 rpm for 20 minutes at 37 °C. During incubation, the lacrimal suspension was shaken 60 times every 5 minutes to completely disrupt the cell clumps. The cells were collected by centrifugation at 1700 rpm for 3 minutes. The cell pellet was resuspended in 1 mL of TrypLE and transferred to a 1.5 mL tube. The cell suspension was incubated in a thermoshaker at 1500 rpm for 30 minutes at 37 °C. The cell suspension was pipetted 20 times every 10 minutes to completely disrupt the cell clumps. The digestion reaction was quenched by adding 1 mL of DMEM supplemented with 10% FBS, and the cell suspension was applied to a 70 μm cell strainer placed in a new 15 mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. A centrifugation step at 1700 rpm for 3 minutes was performed to collect cells. The cells were resuspended in 1 mL of red blood cell lysis buffer and incubated for 2 minutes at room temperature, after which 4 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 2 mL of 0.04% BSA in DPBS.

10. Liver

(1) Solution preparation

Table 1 Components of Solution I

Component	Final concentration	Volume (100 ml)

1X EBSS without Ca^{2+}/Mg^{2+} (Solarbio, H2045-500)	1X	100 ml
100 mM EGTA (pH=8.0) (NaOH is needed to facilitate resolving)	0.5 mM	0.5 ml
Concentrated hydrochloric acid		30 μl (pH 7.4)

Table 2 Components of Solution II

Component	Final concentration	Volume (100 ml)
1X EBSS with Ca^{2+}/Mg^{2+} (Solarbio, H2025-500)	1X	200 ml
1 M HEPES	10 mM	2.0 ml

Table 3 Components of Solution III

Component	Final concentration	Volume (100 ml)
Solution II	1X	100 ml
25 mg Collagenase II	25 mg	-

(2) Intubation perfusion

The thoracic cavity, abdominal cavity, and thoracoabdominal septum were cut off. The inferior vena cava was clamped with hemostasis forceps. The indwelling needle was inserted into the superior vena cava from the apex of the heart. The peristaltic pump was turned on with the parameter setting at 15 and then paused if the liver was filling. The peristaltic pump was paused. After the portal vein was cut off, the peristaltic pump was turned on again for perfusion with Solution I for 10 minutes, Solution II for 10 minutes and Solution III for 10 minutes (the solutions were preheated at 37 °C).

(3) Dissociation into single cells

After intubation perfusion, the liver was carefully removed and placed into a 10-cm dish containing 10 mL Solution III. Cells were released by pricking with a 1 mL pipette.

The cell suspension, mainly comprising hepatocytes, was transferred into a 50 mL tube, followed by the addition of DMEM-FBS. Subsequent dissociation into single cells was divided into two parts: the cell suspension (mainly including hepatocytes) and backbone tissues (mainly including cholangiocytes, immune cells and stromal cells). (1) The cell suspension was further applied to a 70-μm cell strainer and centrifuged at 50 g for 5 minutes. Cells were further digested using 2 mL of collagenase II supplemented with Y-27632 for 10 minutes and pipetted every 5 minutes, followed by the addition of 4 mL of DMEM-FBS. Subsequently, centrifugation at 50 × g for 5 minutes was performed to collect hepatocytes. (2) Backbone tissues were transferred into a 1-mL tube and cut into small pieces of 2-3 mm. Tissue pieces were further transferred into 15-mL tubes containing 9 mL of collagenase II and incubated for 40 minutes at 37 °C with pipetting 20 times every 20 minutes. Cells were collected by centrifugation at 300 × g for 5 minutes and resuspended in 2 mL of collagenase II supplemented with Y-27632, followed by a 10-minute incubation with pipetting 20 times every 5 minutes. The cell suspension was further applied to a 70 μm cell strainer, and 5 mL of DMEM-FBS was added to wash the cell strainer. Cells were centrifuged at 300 × g for 5 minutes and resuspended in 2 mL of red blood cell lysis buffer (Miltenyi, 130-094-183) for 2 minutes of incubation at room temperature, followed by the addition of 8 mL of PBS. Cells were centrifuged at 300 × g for 5 minutes and resuspended in 3 mL of DMEM-FBS. (3) The cells from the two parts were mixed together at a cell number ratio of 1:1 and centrifuged at 300 × g for 5 minutes. The cells were resuspended in 0.04% BSA in DPBS.

11. Lung

For epithelial cells, the left atrium was removed, and 10 mL of cold PBS was injected into the right ventricle to wash away the blood cells in the lung. Then, the lung was removed, cut into small pieces and incubated in 2.4 mL of collagenase/hyaluronidase at 37 °C for 40 minutes in a thermoshaker with vigorous shaking 60 times every 10 minutes. Digested cells were transferred to a new 15 mL tube, and 5 mL of DMEM supplemented with 10% FBS was added immediately to terminate digestion. Cells were

filtered through a 70-μm cell strainer and then resuspended in 1 mL of red blood cell lysis buffer for 2 minutes at room temperature. Cells were washed once using PBS and filtered through a 40-μm cell strainer. Filtered cells were resuspended in FACS buffer and then stained with 1:500 Epcam-APC/Cyanine7 (BioLegend 118218) on ice for 30 minutes. DAPI (1:100, Sigma) was used to stain the cells for 10 minutes on ice. $Epcam^+$ epithelial cells were sorted with a Sony MA900. For all the cells (immune cell enrichment), the enzyme mixture was prepared by adding 2.4 mL of 1× Buffer S, 100 μL of Enzyme D, and 15 μL of Enzyme A to a gentleMACS™ C tube. Lungs were dissociated into single cells using a lung dissociation kit (Miltenyi, 130-095-927) with a modified protocol. The lungs were dissected into single lobes, which were subsequently injected with 200 μL of enzyme mixture. The lobes were transferred into a gentleMACS™ C tube containing enzyme mixture and cut into two pieces. The tissue pieces were then dissociated into single cells with Dissociator using the 37C_m_LDK_1 program. Cells were collected by a brief centrifugation at 300 × g for 10 seconds. The cell suspension was applied to a 70 μm cell strainer placed in a 10 mL tube, and the cell strainer was washed with 2.5 mL of Buffer S, followed by centrifugation at 300 × g for 10 minutes. The cells were resuspended in 300 μL of red blood cell lysis buffer (Miltenyi, 130-094-183) and incubated for 2 minutes at room temperature. Then, 4 mL of PBS was added, followed by centrifugation at 300 × g for 5 minutes. The above step to remove red blood cells was repeated, followed by a dead cell removal step using a Dead Cell Removal Kit (Miltenyi, 130-090-101). The cells were finally suspended in 300 μL of 0.04% BSA in DPBS.

12. Pancreas

The site at which the hepatic duct and the cystic duct converged was clamped with surgical clamps to block the pathway to the liver and gall bladder after opening the mouse abdomen. A 30G1/2-G needle was inserted from the ampulla on the duodenum, which is the site of convergence of the common duct and the intestine, and then 2 mL of 0.5 mg/mL collagenase P (Roche, 11213857001) was slowly injected. The pancreas was removed and placed in a 15-mL tube containing 8 mL of collagenase P. The tube

was placed in a water bath at 38 °C for 10 minutes. After incubation, the tube was shaken vigorously 40 times within 10 seconds. The digestion was terminated by placing the tube on ice, and 8 mL of DMEM supplemented with 10% FBS was added. A centrifugation step at 800 rpm for 2 minutes was performed to collect the cells. The cells were resuspended in 50 mL of DMEM supplemented with 10% FBS, and islets were isolated by hand-picking under a microscope. The endocrine cells were then digested with TrpLE at 37 °C for 15-20 minutes. Exocrine cells were incubated with TrpLE for 20 minutes and filtered through a 70-μm cell strainer and then resuspended in 1 mL of red blood cell lysis buffer for 2 minutes of incubation at room temperature. Endocrine cells and exocrine cells were mixed at a 1:1 ratio as one sequencing sample.

13. Salivary gland

Salivary glands, including the submandibular and sublingual glands, were removed and minced into small pieces in cold PBS on ice. Tissue pieces were then incubated on a thermoshaker at 37 °C for 30 minutes with vigorous shaking 60 times every 10 minutes using 750 μL of collagenase/hyaluronidase supplemented with DNase I. A centrifugation step at 1700 rpm for 3 minutes was performed to collect cells. Then, 750 μL of TrpLE was added to digest the pellets into single cells on a thermoshaker at 37 °C for 30 minutes. The cells were filtered through a 70 μm cell strainer after termination of digestion and then were resuspended in 1 mL of red blood cell lysis buffer for 2 minutes at room temperature. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 200 μL of 0.04% BSA in DPBS.

14. Skeletal muscle

The left hindlimb muscle was dissected, followed by the removal of all connective tissues. Skeletal muscle was dissociated using a skeletal muscle dissociation kit (Miltenyi, 130-098-305) with a modified protocol. Skeletal muscle (~0.9 g) was cut into small pieces of 2-4 mm in 10-cm dishes and then transferred to a gentleMACS™ C tube containing the enzyme mixture (4.7 mL of DMEM, 200 μL of Enzyme D, 50 μL of Enzyme P, and 36 μL of Enzyme A). Tissue pieces were then dissociated into single

cells with Dissociator using the 37C_mr_SMDK_1 program. After dissociation, the cells were briefly centrifuged at 300 × g for 10 seconds at 4 °C, and 10 mL of DMEM-FBS was added to quench the enzymatic reaction. The cell suspension was then applied to a 100-μm cell strainer placed in a 50-mL tube, and then, the C tube was washed with 5 mL of DMEM-FBS, which was also applied to the cell strainer, followed by a centrifugation step of 300 × g for 20 minutes at room temperature. Red blood cells were lysed by incubation in 2 mL of red blood cell lysis buffer for 2 minutes, followed by the addition of 8 mL of PBS. Cells were collected via a centrifugation step at 300 × g for 5 minutes, resuspended in 1 mL of DMEM-FBS and then applied to a 40-μm cell strainer, followed by centrifugation at 300 × g for 5 minutes. Cells were suspended in 1 mL of DMEM-FBS and then applied to an MS column (Miltenyi, 130-042-201) to remove cell clumps. The cell suspension was applied to a new MS column. Seven milliliters of PBS was added to the cell suspension, followed by centrifugation at 300 × g for 5 minutes. During centrifugation, the latter MS column was washed with 1 mL of PBS twice. The cells were resuspended in 1 mL of PBS and applied to the MS column that had been washed. Seven milliliters of PBS was added to the cell suspension, followed by centrifugation at 300 × g for 5 minutes. The cells were resuspended in 500 μL of 0.04% BSA in DPBS.

15. Spleen

The spleen was dissociated and transferred into a 1.5 mL tube containing the enzyme mixture (750 μL of collagenase/hyaluronidase and 0.75 μL of DNase I) after connective tissue removal. The spleen was cut into small pieces and placed in a thermoshaker for an incubation step at 1500 rpm for 15 minutes at 37 °C. During incubation, the spleen suspension was shaken 60 times every 5 minutes to completely disrupt cell clumps. The cell pellet was resuspended in 750 μL of DMEM supplemented with 10% FBS and applied to a 70-μm cell strainer placed in a new 15-mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. A centrifugation step at 1700 rpm for 3 minutes was performed to collect the cells. The cells were resuspended in 1 mL of red blood cell lysis buffer and incubated for 2 minutes at room temperature,

after which 4 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 2 mL of 0.04% BSA in DPBS.

16. Stomach

The stomach was dissociated and transferred into a 1.5 mL tube containing the enzyme mixture (1.2 mL of collagenase II supplemented with 13 mg dispase II and 1.2 µL of 100 mM Y-27632) after connective tissue removal (especially the attached gastric adventitia on the surface of the stomach and the transparent part of the gastric tissue). The stomach was cut into small pieces and placed in a thermoshaker for an incubation step at 1500 rpm for 15 minutes at 37 °C. During incubation, the stomach suspension was shaken 60 times every 5 minutes to completely disrupt cell clumps. The cell pellet was resuspended in 4 mL of DMEM supplemented with 10% FBS and collected by centrifugation at 300 × g for 5 minutes. The cell pellet was washed with 4 mL of PBS and then collected by centrifugation at 300 × g for 5 minutes. The cell pellet was resuspended in 1 mL of TrypLE (twofold dilution in Advanced DMEM-F12 supplemented with 1% penicillin–streptomycin, 0.2% primocin, 1% HEPES and 1% GlutaMAX) and incubated in an incubator for 10 minutes at 37 °C. The cell suspension was pipetted 20 times every 5 minutes to completely disrupt the cell clumps. The digestion reaction was quenched by adding 4 mL of DMEM supplemented with 10% FBS, and the cell suspension was applied to a 70-µm cell strainer placed in a new 15-mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. Cells were centrifuged at 300 × g for 5 minutes and resuspended in 100 µL of 0.04% BSA in DPBS.

17. Thymus

The thymus was dissociated and transferred into a 1.5 mL tube containing the enzyme mixture (750 µL of collagenase/hyaluronidase and 0.75 µL of DNase I) after connective tissue removal. The thymus was cut into small pieces and placed in a thermoshaker for an incubation step at 1500 rpm for 15 minutes at 37 °C. During incubation, the thymus suspension was shaken 60 times every 5 minutes to completely disrupt cell clumps. The

cell pellet was resuspended in 750 µL of DMEM supplemented with 10% FBS and applied to a 70-µm cell strainer placed in a new 15-mL tube. The cell strainer was then washed with 2 mL of DMEM supplemented with 10% FBS. A centrifugation step at 1700rpm for 3 minutes was performed to collect the cells. Cells were resuspended in 1 mL of red blood cell lysis buffer and incubated for 2 minutes at room temperature, after which 4 mL of PBS was added to the cell suspension. The cells were centrifuged at 300 × g for 5 minutes and resuspended in 2 mL of 0.04% BSA in DPBS.

Supplementary Tables

Supplementary Table 1. General information for the atlas. This file includes six sheets presenting the information about the cell number of each cell type, each tissue and each condition, the biological replicate, and the cell markers of each cell type (avg_log_2FC > 0.5 and *p_val_adj* < 0.05). Cell markers were defined by Wilcoxon Rank Sum test and *P* values were adjusted by the Bonferroni correction using the *FindAllMarkers* function implanted in Seurat.

Supplementary Table 2. Cell proportions. This file includes four sheets presenting fold changes in the proportions of all cell types and immune cell types based on the three comparisons of MS vs. FS, MC vs. MS and FD vs. FS.

Supplementary Table 3. Differentially expressed genes (DEGs). This file includes four sheets presenting the DEG information for each cell type. DEGs were defined by Wilcoxon Rank Sum test and *P* values were adjusted by the Bonferroni correction using the *FindMarkers* function implanted in Seurat.

Supplementary Table 4. Significantly enriched biological pathways. This file includes one sheet that presents significantly enriched biological pathways ($p < 0.01$ and $q < 0.01$) based on the DEGs in each cell type between MS and FS, MC and MS, FD and FS mice. Significantly enriched biological pathways were defined by the hypergeometric distribution and *P* values were adjusted by the Benjamini-Hochberg method (BH) by using *EnrichGO* function implanted in the clusterProfiler.

Supplementary Table 5. Information for scRNA-seq data of FOD, FO and MCD conditions. This file includes five sheets presenting the information about the cell number of each cell type, each tissue and each condition, the biological replicate of FOD, FO and MCD condition.

Supplementary Table 6. Androgen-associated sex-biased DEGs (AASB-DEGs). This file includes four sheets presenting AASB-DEGs in each cell type, tissue-shared AASB-DEGs and their involved biological pathways. DEGs were defined by Wilcoxon

Rank Sum test and *P* values were adjusted by the Bonferroni correction using the *FindMarkers* function implanted in Seurat.

Supplementary Table 7. Sex-biased diseases and their risk genes. This table includes three sheets related to sex-biased and sex-comparable diseases, their risk genes and disease-shared risk genes. The proportion test was performed and *P* values were adjusted by the Bonferroni correction compare the disease incidence rates between males and females. The GWAS summary statistics of each disease phenotype were used as input to calculate the FDR values (the Benjamini–Hochberg) for SNPs to define risk genes.

Supplementary Table 8. Cell-type enrichment for sex-biased diseases. This file includes three sheets presenting the disease enrichment score of each disease in each cell type, the top 10 cell types with the highest enrichment scores for each disease and each disease group.