Practical Clinical Pathology

‘Minimally-invasive’ Phenotyping: for mice GEM etc

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Discussion Plan

♦ Clinical Pathology tests, what they assess, how to get useful results:
  1. Specimen collection & processing - issues & options
  2. Practical hematology - mostly mice
  3. Practical chemistry - mostly mice
  4. A little urinalysis - mostly mice
     • Examples, problems, recommendations

♦ Emphasis: practical, reproducible, less invasive, less expensive.

Conclusions

1. Minimally invasive and relatively inexpensive tests can provide valuable and valid data.
   • Design studies to take advantage of these
2. (Mis) handling of animals or specimens can lead to invalid results (bad data or false ‘phenotypes’), or big SD (useless data)...  
   • Learn about the tests & complications 1st
   • Standardize protocols - they must be repeatable.
3. Concurrent Relevant controls are essential.

Practical Clinical Pathology

♦ Clinical (survival) specimens
  ♦ Blood
    - Whole blood → Hematology
    - Serum/plasma → Clinical Chemistry
  ♦ Mouse data & protocols at
    - MPD https://phenome.jax.org/
    - IMPRESS http://www.mousephenotype.org/impress
  ♦ Urine
  ♦ Feces
  ♦ Other fluids, cells

Practical Clinical Pathology

♦ Survival (or terminal) collections
  ♦ Blood
    - Glucose by glucometers
    - Hematology, chemistry, proteomics
  ♦ Urine
    - SG, Glucose, PH, metabolomics
  ♦ Feces
    - Hema occult, fecal fat, parasites

Value of Clinical Pathology

→ DATA usually quantitative/objective
→ Animals can/should survive
→ Relevant to many phenotypes

<table>
<thead>
<tr>
<th>Hematology</th>
<th>Chemistry</th>
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<td>Inflammation</td>
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<td>Thrombocytopenia</td>
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<td>Neoplasia</td>
<td>Carbohydrate &amp; Lipid metabolism</td>
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<td></td>
<td>Neoplasia</td>
</tr>
</tbody>
</table>

Glucose

♦ Glycemia via Glucometers
  • Cage side
  • $< 100. / glucometer
  • $2-2.50 per strip!
  • <10ul / sample
  • Downloadable data?
  • Mouse wrangling & stress
    - Before or after other procedures
    - Including cage changing
    - Fasting ?
    - Glucose challenge, meal challenge
    - Insulin response ?

Glucometers

♦ Accu-Chek® Roche
♦ OneTouch® Lifescan
♦ Vs Vetace

- Glucometer Volume < 10ul
- Ease of use
- Data downloading
- Validated?
  Forbes & al 2008
Blood Collection Variables to standardize AND report...

- PRE analytical factors:
  - Collection Site;
  - Anesthesia;
  - Time of day;
  - Fasting (?): glucose, hemococentration, cortisol;
  - Mouse handling (cortisol, stress leukocytosis, etc);
  - Specimen handling (evaporation, hemolysis, storage);

- Analytical factors:
  - Instrument/test method(s).

**How often do you see all of these in protocols, and methods sections of publications...**

How/where to bleed a mouse

- Peripheral (survival) sites
  - RetroOrbital sinus (not plexus)
  - 'tail clip' (= amputation)
  - Facial vessels - Cheek? Chin?
  - Tail cut - lateral in mice
  - Saphenous (medial > lateral marginal)
  - Usually mixed arterial and venous

- Tissue damage - do NOT squeeze to get more blood...

MOUSE blood collection sites

- RetroOrbital/bulbar RB; Facial vessels FV:
  - Up to 0.5ml? (- operator experience/expertise)
  - Which facial vessels??
  - Arteries usually run with veins...

- Lateral Tail Vein, Saphenous vessels
  - <0.2ml
  - Effects of squeezing © aka milking leg, tail: expect hemolysis, CK, LDH, K etc...

- Cardiac/terminal
  - Up to 1.5ml?
  - Mouse size, operator experience/expertise...

FACE blood collection

- Facial/Cheek Blood collection

  - Veins with arteries
  - Cheek (low)
    - Facial vessels ("submandibular v")
  - Cheek (high)
    - Inferior palpebral or transverse facial vessels?
    - Superficial temporal and maxillary vv?
  - Chin
    - Submental or inferior labia VV?

FYI ‘Facial’ Blood collections

- Facial Vessels
  - Veins with arteries
    - Cheek (low)
      - Facial vessels ("submandibular v")
    - Cheek (high)
      - Inferior palpebral or transverse facial vessels?
      - Superficial temporal and maxillary vv?
    - Chin
      - Submental or inferior labia VV?

FYI Facial Vessels

- Golde et al 2005
  - Which vessels ?? 'submandibular' ?
  - Fig 3a: ‘Positioning and poking the cheek with the lancet...’

Submental or inferior labial ‘vein’?

- Target, 1-2 mm from midline, for vascular access (arrows) mouse.

FYI Facial vessel blood collections...

- Cheek? or Chin?
- Vessels - not just veins...
- What vessels do you think you are aiming for? or hitting?
  - Check by dissection? Post mortem?
RetroOrbital Venous Sinus or Plexus?

- Blood collection, or injection.
- (AFTER Approval by IACUC/ethical committee)

A. Medial canthus ?
B. Lateral canthus ?
C. Dorsal to the eye


Facial ‘Vein’ Vs Retrobulbar

Teilmann et al. 2014.
- B6 male, 5mo, n= 12/g
- No anesthesia
- RB 75ul capillary
- FV lancet

FV bled mice had elevated corticosterone
FV bled had more wt loss than RB bled
Both had tissue damage

Collection options - MICE

https://norecopa.no/films-and-slide-shows/mouse
https://www.idexxbioanalytics.com/hubfs/Discovery-
Resource%20Materials/Patho/Blood%20Sample%20Collection%20
Guide%20v6.pdf etc

MOUSE Collection site options - Max blood volume
Retroorbital SINUS (retrobulbar) <0.5ml?
CHIN Submental/ Inferior labial vv <0.2ml
CHEEK Submandibular/Facial Vein <0.05ml (50ul)
Superficial temporal ? <0.5ml?
Lateral Saphenous <0.2ml
Tail tip (amputation/ mixed blood) <0.2ml
Cardiac / terminal 1ml / more?
Caudal Vena Cava / terminal ~1ml

REPEAT collection effects
Raabe et al 2011.
- Weekly collections
- B6 male /female, 10-14wo, n ~20/g
- 15%, 20%, or 25% of estimated total blood volume (TBV) collected once weekly x 6 wk.
- Fentanyl / retrobulbar

EXPECT REGENERATIVE RESPONSES BUT:
- Up to 25% TBV was collected once weekly from female mice x 6 wk;
- Up to 15% TBV was collected once weekly from male mice x 6 wk;
- without weight loss, behavioral changes, or clinically significant anemia.

Collection site Matters ?
Regan et al 2016

BEWARE of Dr. Google....

FYI Blood Collection - Tail

- Rodent tail, cross section
- vessels used for blood collection (injections).
- Rats, Mice
- Hamsters, Gerbils

THOMAS DEERINCK, NCMIR/ SCIENCE PHOTO LIBRARY
- Caption: “Mouse tail. Light micrograph of a cross-section through a mouse's tail. At centre is the tail vein, which contains red blood cells, whose nuclei have been stained blue. A smaller vein (green squiggle) is seen at top centre. The two white circles above the central vein are cartilage of the vertebral column, and each has a large nerve (orange speckled circle) adjacent to it. The large brown ovoid structures are muscles and tendons....”
  http://www.sciencephoto.com/media/385881/view

Collection site Matters ?
Retroorbital vs Facial vs other

- Whatever you’re good at
- Facial Better than retroorbital specimens?

Saphenous

- Lateral or medial
- Medial saphenous
- Lateral marginal or saphenous
- Right handed operator holds mouse with left hand →
  - <50 ul
  - Needle or lancet to nick

Peripheral Collection without anesthesia

- Facial
- Leg
- Tail
  - Pick 1
  - Practice, train
  - Report it accurately

How much blood in a mouse?

- Varies with mouse size/weight & collection frequency (Calculation method)
- Total Blood volume calculation
  - 55 - 70 ml/kg body weight
  - 5.5 - 7% of body weight
  - 1.1 to 2.8 ml = TOTAL blood in 20-40g mice

How much can you take?

- McGuill 1989 (ILAR)
  - Emphasizing survival ..
  - = basis for many guidelines / recommendations
    - 150-600ul ~ 15% blood volume → wait 4 weeks
    - 100-400ul ~ 10% → wait 2 weeks
    - 75-300ul ~ 7.5% → wait 1 week
    - 10-40ul ~ 1% → Daily x ??
  - Bleeding can be difficult to control...
  - Include concurrent relevant controls!

How much can you take?

- Diehl & al. 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes.
  - Bleeding can be difficult to control...
  - Include concurrent relevant controls!

ABL Acute Blood Loss Effects

Marx et al. 2015.
- C57BL/6j
  - 0 Rx, DM/STZ or DSS Rx
  - Retroorbital /Isoflurane
  - -15-16% BV - 230-300ul

Marx et al. 2015. COMP MED https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4485629/

Site matters

Abatan et al. 2008
- ‘Modified tail-clip’
  - Tail tip amputation + EDTA capillary tube
  - Vs saphenous venipuncture
  - 4 collections in 1 wk
  - ~ 40ul
  - Tail clip yielded Higher WBC, NE, LY

Experience matters too

- Experienced, quick operators → lower corticosterone
Collection site & WBC

Doeing & al 2003

CONCLUSION
→ Use the same site within and between studies...
→ Report site etc details

Collection site & Chemistry

Fernandez & al 2010

- "Submandibular - vein" vs Retroorbital "Plexus"
- (Cheek vs Retroorbital sinus)

CONCLUSION
→ Use the same site within and between studies...
→ Report site etc details accurately...

Blood TUBES

- Anticoagulant
  - Wet
  - Dry
- Volume
  - Tube
  - Anticoagulant
- Shape
  - Capillary tube?
  - Shape for instrument?
  - Spill proof?

→ Request sample tubes!
→ What works best for your species collections, instruments ...
→ Probably NOT Eppendorf...

Blood Tubes

- For hematology
  - Anticoagulated whole blood
    - K₂EDTA – usually recommended
    - lavender / pink
    - BD microtainer
    - Better morphology
    - Inhibits bacteria
    - Dry - sprayed on does not dilute
    - Wet - dilutes small specimens
    - Underfilling shrinks RBC (HMCV) due hypertonicity

Blood Tubes

- For hematology
  - Anticoagulated whole blood
    - Heparins
    - Green top tubes
      - Morphology & platelet clumps → PLT
        - Interferes with some immunoassays
  - plasma chemistry from same specimen!

Why use Separator Tubes?

- MUST allow time to clot
  - ~ 30 min.

NOTE more & cleaner serum

Hematology Analysis

MANUAL

- WBC Diff (%)
  - Always Smear
  - only 2.5ul!

- Hemacytometer
  - <40ul → RBC WBC PLT
  - WBC # not type
  - Still need smear to assess morphology + differential

Location, location, location:

Shock. 2014. Mella & Al.

- Cytokine concentrations are dependent on blood sampling site.
- proinflammatory and anti-inflammatory cytokine concentrations are dramatically elevated when drawn centrally from the heart compared with collection from peripheral locations such as the facial vein.
- It is critical for publications to document the sampling location when evaluating plasma cytokines and attempting to compare studies.

2019 JH Phenotyping

Educational Use Only
Hematology Analysis

- Automated
  - $20,000 - $60,000 - impedance based
  - > $150,000 - FACS based
  - + reagents + service
  - 20 - 200uL blood required for CBC
- FACS (flow cytometry)
  - More specialized - Immunophenotyping
  - Lymphocyte types
  - Developmental stages

CBC

- Complete blood count
- White cells
- Red cells
- Platelets

WBC

- Cell counts #/volume
- Neutrophils
- Lymphocytes
- Monocytes
- Eosinophils
- Basophils
- Monocytes

Hematology of Swiss mice

- Restell et al. 2014
  - A. Neutrophil
  - B. Lymphocyte
  - C. Monocyte
  - D. Eosinophil
  - E. Polychromatic erythrocyte
  - F. Platelets

IDEXX ProCyte Dx®

- Impedance
- + Laser Flow cytometry + Fluorescence
- Mini version of Siemen Sysmex technology (& LaserCyte® technology)
  - 30uL !!
  - Validated for 10 sp
  - Reticulocytes
  - Platelets

IDEXX ProCyte Dx® (& LaserCyte®)

- Complete blood count
- White cells
- Red cells
- Platelets

Advia, Celldyn etc

- FACs based
- >$200,000
- Hi maintenance $,
- Hier specimen volumes
- Advia
  - Myeloperoxidase channel
  - LUC
- $$$ options to digitally assess and image capture from smears !!

MOUSE HEMATOLOGY (CBC)

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<th>Mean</th>
<th>SD</th>
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<td>NRBC (K/µL)</td>
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<td>0.0</td>
<td>1122</td>
</tr>
</tbody>
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Our results from mice over ~1yr Excluding results with ‘error’ – avail online at:
- https://researchanimalresources.jhu.edu/services/rodent-services/rodent-phenotyping
- http://mcp.bs.jhmi.edu/phenotyping-core-testing

Abnormal CBC results...

A Few Drops?
- Smear <5ul
- Glucose <10ul
- CBC <30ul
  (impedance system)
- PCV/TS - clinical
  - Hemacytometer

CBC (complete blood count)
- USE concurrent relevant controls
  - Strain, sex, age differences
  - Environment/pathogen status differences
- HCD, reference ranges, published ‘normals’
  - CHECK DATES - 1960’s probably not relevant
  - MPD http://phenome.jax.org/
    -> Kile, & al. (2003) (Abbot Cell Dyn)
    -> Peters & al. (2002) (Bayer - Siemens Advia)
- Data analysis
  - Use absolute numbers for data analysis (statistics)
    - i.e. NOT %’s (though you will see this in literature)

Hematology - Mice
- Mouse RBC
  - Hi RBC - 7-11 x10^6/ul
  - Hi HCT PCV
  - Lifespan - many circulations ~40-50d
  - Polychromasia anisocytosis retics are WNL
    - More retics in young - stabilize by 2-3mo
  - 4-7u diam on smear
  - Na ~ 13; K ~ 118 mmol/L (~ hu)
    - Hemolysis → serum/plasma

Comparative RBC lifespans
- Humans 100-120 d
- Dogs 100-115 d
- Rats 45-50 d
- Mice 40-50 d
- Anemia of chronic dz develops quicker with shorter RBC lifespan

RBC - Mice
- Strain
- Sex

ANEMIA: The repeat blood collection phenotype

Regenerative anemia
- HCT, RBC, MCV, MCHC
- size MCV
- Microcytic, hypochromic,
- fewer, bigger, paler
- RDW may decrease
  - Generally increased size (MCV)
  - With fewer small mature cells
- Concurrent controls should have similar volumes drawn

ANEMIA: A real phenotype
NON Regenerative anemia
- HCT, RBC, MCV
- MCH, MCHC
- Microcytic, (hyperchromic - relative)
- fewer older cells
- RDW should decrease
  - Generally decreased size (MCV)
  - With fewer large immature cells
- Concurrent controls should have similar volumes drawn

Reticulocytes (retics)
- Immature RBC
- ~1% of human RBC
- develop in marrow
- circulate ~1d → Mature RBC
- reticular (mesh-like) network of ribosomal RNA seen with certain stains e.g. NMB → new methylene blue.

(HB = oxidized Hemoglobin Hb)
HCT - Mice
- Strain
- Sex
- Age
- Similar to PCV
- Calculated
- ~50

MCV - mean corpuscular volume
- Strain
- Sex
- Size in femtoliters

RDW = red cell distribution width
- Strain
- Sex
- How variable is cell size?

MCHC = mean corpuscular hemoglobin concentration
- Strain
- Sex
- How concentrated is the hemoglobin? g/dl

MCH = mean corpuscular hemoglobin
- Strain
- Sex
- How much hemoglobin per cell? pg

PLT - Platelets
- Strain
- Sex
- Many more than most species!
- STRAIN Variation 300-2000 x10^6/ul
- 1-4u
- PDW > RDW
- Half life ~ 5d (shorter in C3H, CBA)
- Thrombocytopenia? Check for CLUMPS
- Megakaryocytes (20-30u)
  - Expected in spleen & marrow
  - Ploidy usually 16N (32N in C3H)
  - 32N in regeneration
  - Emperipolesis - cell transit thru mega etc

Hematology - Mice
- WBC - report absolute counts!
  - Expect 3-5000/ul in clean young mice;
  - M>F, w age
  - LYMPHOCYTE LEAD ~2-3 x NE
  - LUC <5%
  - NE (�� by IL3, GCSF, IL6, GMCSF etc)
  - Eo << 1K (�� by IL3, IL5 etc)
  - Baso rare on smears
    - Lobulated nuclei vs tissue mast cells
  - Mono < 1K (�� by GCSF, GMCSF, IL3 etc)

Hematology - Mice
- WBC (3,000 - 10,000/ul) - clean mice
  - M > F, increase with age
  - (~ 5000 in really clean young mice)
  - (~ 1000 in clean scid, nude, NOD scid, NSG)
- Lymphocytes (~60-90% -2-3x neutrophils)
  - Lower in clean scid, nude, NOD scid, NSG
- Neutrophils (~10-30%)
  - Higher in scid, nude, NOD scid, NSG
Hematology - Mice

- Mono, Eos, Baso (< 1 x10^3/ul, <2%)
  - Eo: by parasites, allergens
    - IL3, IL5 etc
  - Baso: usually rare
    - tissue mast cells?
  - Mono: by chronic infection
    - GCSF, GMCSF, IL3 etc
  - LUC < 5% (large unidentified cells)

Machines cannot (correctly) identify abnormal cells! → ALWAYS Smear
  → stain & evaluate hi LUC etc abnormalities

WBC - Mice

- Strain
- Sex
- Age
- MPD
  - Jax4
  - 8wo, 16wo
  - http://phenome.jax.org/

WBC - older Mice

- Strain
- Sex
- Age
- MPD
  - Peters 4
  - 6-24 mo
  - http://phenome.jax.org/

Common Leukocyte response patterns (mice)

- Acute Inflammation
  - WBC: Ne (or depletion) neutrophils
  - Immature neutrophils

- Chronic Inflammation
  - WBC: Ne, Ly, Mo (RBC in anemia of chronic disease)

- Excitement / Catecholamine
  - Ne: proportional

- Stress / Corticosterone
  - Ne: hypersegmentation, Ly: Eo

- Allergy
  - hypersensitivity
  - parasites

61008

- Il10 tm
- Neutrophils
  - Maturity?
- Monocytes
- lymphocyte

61021

- Il10 tm
- Neutrophils
  - Maturity?
- platelets

61021

- Il10 tm
- Marrow
- M:E?
- ??

Educational Use Only
Basophil vs Mast cell
Systemic mastocytosis
Mouse mast cell tumor
Don’t squeeze
  Tissue contamination

Basophil vs Mast cell
Systemic mastocytosis
Mouse mast cell tumor
Neutrophils
Eosinophil
Lymphocytes

Some Factors that affect hematology test results

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<thead>
<tr>
<th>Physiology</th>
<th>Collection</th>
<th>Analysis</th>
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<tbody>
<tr>
<td>Age</td>
<td>Study design</td>
<td>Sample quality (hemolysis, lipemia, clots)</td>
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<tr>
<td>Sex</td>
<td>Movement of cages</td>
<td>Sample storage &amp; handling</td>
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<tr>
<td>Strain</td>
<td>Prior handling/dosing</td>
<td>Instrumentation</td>
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<td>Fasting/Fed status</td>
<td>Site of Collection</td>
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<tr>
<td>Concurrent illness</td>
<td>Anticoagulant</td>
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<tr>
<td>Other experimental Procedures</td>
<td>Anticoagulant:Blood ratio</td>
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</table>


Fasting
Fasted rodents usually don’t drink either → Hemoconcentration
Expect some changes in hematology + serum chemistry
  ©Body wt
  ©Glucose, ©Triglyceride, ©Cholesterol
  ©ALKP and ALT
  Increased © Creatinine

Artifacts in Mouse (etc) Hematology

<table>
<thead>
<tr>
<th>Decreased RBC</th>
<th>CLOTS</th>
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<td>UNDERfilled Collection Tube</td>
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<table>
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<th>Increased MCHC</th>
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<td>Heinz Bodies</td>
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<table>
<thead>
<tr>
<th>Increased eos</th>
<th>Platelet clumps - on Advia / technicon (myeloperoxidase)</th>
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<table>
<thead>
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<th>Decreased PLT</th>
<th>CLOTS</th>
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<tr>
<td>Difficult sample collections</td>
<td>Platelet clumps</td>
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<tr>
<td>Improper gate settings</td>
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<table>
<thead>
<tr>
<th>Prolonged clot times</th>
<th>CLOTS</th>
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<td>Difficult sample collections</td>
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<td>UNDERfilled collection tube</td>
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<tr>
<td>He HCT (+ XS anticoagulant)</td>
<td>Poor handling</td>
</tr>
</tbody>
</table>

One word about Hematology reference ranges (intervals):
LIES.

 Lies, Damn Lies, and Reference Intervals (or Hysterical Control Values for Clinical Pathology Data).

Concurrent relevant controls are critical.

RODENT COAGULATION
Blood clots quickly BUT Resistant to thrombus formation....
Results affected by collection techniques
Typical coag biomarkers not so sensitive or specific....
Endpoints not standardized /validated....
  Some labs get good at tail bleeding time...
  (influenced by strain anatomic variations Hoover Plow & al)

Clinical Chemistry Equipment
Serum or plasma specimens
  Liver Function ?
  Kidney Function
  Electrolytes
  Lipid panel
  General panel
  Custom panels

Whole blood analyses with hand held (cartridge) or dry systems
  © Small volumes
  © Validated for mice ?
  Improving ?

Some Chemistry Panels

<table>
<thead>
<tr>
<th>Organ System (?)</th>
<th>Tests</th>
<th>Serum plasma</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>AST ALT BIL</td>
<td>100ul &gt;200ul</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>AST ALT LDH AP Alb TP Glu Tbil Dbil</td>
<td>150ul &gt;300ul</td>
<td></td>
</tr>
<tr>
<td>Kid</td>
<td>BUN Creat</td>
<td>100ul &gt;200ul</td>
<td></td>
</tr>
<tr>
<td>Kid</td>
<td>BUN Creat Alb TP K CL Na Ca</td>
<td>250ul &gt;500ul</td>
<td></td>
</tr>
<tr>
<td>Mini</td>
<td>ALT BUN Glu TP</td>
<td>100ul &gt;200ul</td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>Chol Trig HDL (LDL)</td>
<td>150ul &gt;300ul</td>
<td></td>
</tr>
<tr>
<td>Lytes</td>
<td>Na K CL</td>
<td>250ul &gt;500ul</td>
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  ♦ Prioritize tests!
  ♦ Customize panels

Educational Use Only
Liver relevant tests - Mice

- ALT - Liver - also heart, testicular injury
  - by MHV, helicobacters, LDHV - MNV?
- AST - Liver & hemolysis - not so specific
- LDH - Liver heart hemolysis - less specific
- SDH - sensitive & specific IF AVAIL
  - short half life & unstable

Cholestasis

- AP - also intestine & kidney
- GGT - insensitive in mice, rats -
  - Often undetectable - significant if elevated?
- Bilirubin (tbili) ....

Enzymes

ALT Alanine amino transferase
- transfer of α amino group of alanine to α ketoglutaric acid in formation of pyruvic & glutamic acid.
- Fairly specific for hepatocyte injury in dogs cats
  - Usually ALT > AST in liver disease
  - by bile duct obstruction w hepatocyte damage
  - Not so useful for liver in large animals, including pigs,
  - Also in muscle - w trauma, musc dystrophy etc likely cause of in NHP - check CK & LDH
  - Also w hypoxia
  - 0-Low is OK in some species
- Serum half-life 1- few days
  - Only 5hr in Rabbits!!!

AST Aspartate aminotransferase
- Aka Glutamic oxaloacetic transaminase, GOT
  - transfer of α amino group of aspartic acid to α ketoglutaric acid, in formation of oxaloacetic acid & glutamic acid.
  - Indicator of liver and/or muscle injury in large and small animals.
  - In cytoplasm & mitochondria
  - Skeletal muscle > liver, cardiac muscle > kidney, brain - also RBC’s
- by hemolysis

AP, ALP, SAP Alkaline phosphatase
- hydrolyze various phosphate esters at alkaline pH in presence of zinc & magnesium ions.
- different isoenzymes + Isoforms
  - liver-ALP (L-ALP),
    - in Dog Indicates cholestasis, before bilirubin
  - in cats - specific indicator of liver disease,
  - corticosteroid-ALP (C-ALP, only dogs, Humans?),
  - bone-ALP (B-ALP) and
  - intestinal-ALP (I-ALP) - esp rats/mice w inanition
  - in large animals non specific

AP, ALP, SAP Alkaline phosphatase
- Is it usually higher or lower in young (growing) animals?
- Is it usually higher or lower in enteric disease e.g. parasitism?
- Which anticoagulant is better to obtain plasma samples for clinical chemistry and why?
  a) EDTA
  b) Lithium Heparin
  c) Sodium Heparin

LDH Lactate dehydrogenase
- catalyzes lactate to pyruvate
- 5 isoenzymes (LDH1-5)
- not tissue-specific
  - Liver,
  - Heart (MI etc heart damage)
  - Skeletal muscle
- RBC’s - LDH by HEMOLYSIS !
  - LDHV persistently infects monocyte macrophages that normally clear LDH etc enzymes → persistent elevations
  - COMMON biological contaminant

SDH Sorbitol dehydrogenase
- catalyzes fructose to sorbitol
- highest concentration in liver.
- Specific indicator of liver disease in all species
  - SO preferred test for DILI = drug induced liver injury
  - Increases within 24 hours of liver injury
  - Used in rats/tox, also large animals
  - Best? liver test for pigs etc large animals
  - ALT AST unreliable
- Not stable - Handle with care!
  - Short half life (<4 hours in dog, rabbit).
  - ~5 hours at room temperature,
  - 24 hours at 4 C
  - several days frozen

GLDH Glutamate dehydrogenase
- Considered very Liver specific
- Not so widely available but used
  - especially in Large animals and for DILI in Tox - Rat dog NHP
  - Some exotics
Enzymes

Gamma-glutamyl transferase - GGT
= Gamma-glutamyl transpeptidase - GGTP
- important in glutathione metabolism, amino acid absorption and protection against oxidant injury.
- Liver = main source (esp biliary epithelium), sensitive indicator of cholestasis
- should correlate with ALP
- Too low to be useful in mice & rats
- Also in Pancreas, gastrointestinal tract, kidney proximal tubules
- usually shed into ducts lumen
- RATS MICE - often below measurable ranges

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CK
Creatine Kinase
- Aka Creatine phosphokinase CPK
- MUSCLE
- most widely used enzyme for evaluation of neuromuscular disease.
- CK MB isoenzyme - more cardiac specific
- Very short half life, < 1 hour in in some sp
- increases quickly (peaks at 6-12 hours)
- Normalizes 24-48 hours after acute, transient injury.
- Persistent injury will maintain high CK
- stable for 7 days at 4 C; 1 month at -25 C.

Amylase lipase
- Insensitive & non specific indicators of pancreatitis (3-4x inc should be pancreatitis)
- by  GFR dt reduced clearance
- Gastroenteritis, abdominal surgery
- Liver disease
- neoplasia

Which is most SPECIFIC for liver and liver damage?
Which is most useful AND widely available = Practical?
a) ALT Alanine amino transferase
b) AP, ALP, SAP Alkaline phosphatase
c) AST Aspartate aminotransferase
d) CK Creatine Kinase
e) Gamma-glutamyl transferase - GGT
f) LDH Lactate dehydrogenase
g) SDH Sorbitol dehydrogenase

Which are elevated by muscle damage?
a) ALT Alanine amino transferase
b) AP, ALP, SAP Alkaline phosphatase
c) AST Aspartate aminotransferase
d) CK Creatine Kinase
e) Gamma-glutamyl transferase - GGT
f) LDH Lactate dehydrogenase
g) SDH Sorbitol dehydrogenase

Which are elevated by biliary damage?
a) ALT Alanine amino transferase
b) AP, ALP, SAP Alkaline phosphatase
c) AST Aspartate aminotransferase
d) CK Creatine Kinase
e) Gamma-glutamyl transferase - GGT
f) LDH Lactate dehydrogenase
g) SDH Sorbitol dehydrogenase

Which are elevated by hemolysis?
a) ALT Alanine amino transferase
b) AP, ALP, SAP Alkaline phosphatase
c) AST Aspartate aminotransferase
d) CK Creatine Kinase
e) Gamma-glutamyl transferase - GGT
f) LDH Lactate dehydrogenase
g) SDH Sorbitol dehydrogenase
h) Which electrolytes ???

Kidney relevant tests - Mice, rats

BUN - slow
- Various contributors to elevation
- Creatinine - slow
- > 70% loss to get convincing increases
- Uric acid - sometimes useful
  - E.g. PKD! Diabetic nephropathy
- SDMA = new kidney biomarker ??
  - Ca, K

Urine
- SG? - 50uL (not so easy unless PU PD)
- Albumin/microalbuminuria - special instruments
  - Expect proteinuria in rodents rabbits (a, b globulins)
  - Protein losing nephropathy, diabetic nephropathy
- UPC Urine protein: creatinine
  - Collagen IV? Not easy to measure yet
- Kidney Injury Molecule-1 (KIM-1) not so available
DSS BUN Supportive RX

Fluid therapy before and after DSS reduced improved BUN

Electrolytes

- 250ul plasma or serum
- ~500ul blood!
- Na K Cl Ca P (Mg)
  - expect hi CA > 14 in Rabbit

Significant changes
- Near death
- End stage Kidney disease

Will this test tell you something you didn’t know?
Are there more useful tests for that blood?

Cardiac ♥ biomarkers

- Not just LDH & CK anymore
- Troponins (I or T are relevant)
  - ischemia injury marker
  - in renal damage dt clearance
- BNP, BT ProBNP - myofiber stretching
- ANP trial natriuretic peptide

Utility in rodent models?

Lipid Profiles in mice etc

- Mice, rats
  - Cholesterol
  - Triglyceride
  - HDL
  - ‘Non HDL’ cholesterol

- Strain
- Age
- Diet

USE MPD!

HDL Dominant species are atherosclerosis resistant LDL measurement usually not useful ...

The problem with Rodent Lipoproteins ...

- LDL > HDL in Guinea pigs & humans
- HDL >> LDL in mice & rats

Beware of automated analysers
- DO report ‘HDL + Non HDL cholesterol’
- DON’T report or believe reported rat or mouse LDL
  - Check methods!

Fernandez & Volek 2006

Mouse Cholesterol

- Strain
- Sex
- Study

MPD
- 5 study composite

Total Cholesterol and HDL

- mg/DL
- 4wo, 8wo

HDL

- Strain
- Sex
- Study

MPD
- 5 study composite

Mouse

Cholesterol

MPD
http://phenome.jax.org/

HDL

MPD
http://phenome.jax.org/

Total Cholesterol and HDL

http://phenome.jax.org/
Total Cholesterol and HDL

- 8w0, 16wo
- 4wo, 8wo

Triglycerides

- MPD
  - 5 study composite
  - Strain
  - Sex
- Consider Impacts of
  - Mixed backgrounds
  - Mixing data from both sexes

Practical tests for Diabetes

- Blood glucose (> 250 g/dl, >300g/dl)
- Cage side testing, expert operator
- Gluc > 200 is unsurprising after transportation, examination etc stressors...

- Fasting blood glucose - necessary?
  - 4hr food withhold?
  - Or plan bleeds 1-3pm? Normally they would be less active and not eating since dawn/lights on...
  - Mice dehydrate quickly esp when sick
- Urine glucose - dipstick
- IV or (IP in mice) glucose tolerance test

Tests for Diabetes in mice

<table>
<thead>
<tr>
<th>OGTT (oral glucose challenge)</th>
<th>IGT (IV or IP glucose challenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Useful for screening</td>
<td>More sensitive / reproducible than</td>
</tr>
<tr>
<td>More sensitive than fasting blood glucose</td>
<td>OGTT</td>
</tr>
<tr>
<td>Poor reproducibility</td>
<td>Indirect test of insulin sensitivity</td>
</tr>
<tr>
<td>Should be confirmed</td>
<td>Glucose disappearance rate</td>
</tr>
</tbody>
</table>

EG by (hyperinsulinemic ) CLAMP

- Not so practical in mice
  - Surgical catheterization, infusion, repeat bleeding
  - Assesses insulin resistance (glucose disposal)
  - Clamp = glucose infusion rate to maintain euglycemia, in face of continuous insulin challenge (40mU/m2/min)
  - Watch Potassium...

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More test options for Diabetes in Mice etc: Glucose, Insulin, AGE’s

- Insulin levels & Insulin response
- AGE’s - Advanced Glycosylation End product
- Hb1a - glycated hemoglobin
  - Useful in rodents?
  - Is fructosamine a useful surrogate marker?

Clinical Chemistry & Mouse Handling

- E.g.
  - Cage changing
  - Weighing
  - Injections / collections (glucose tolerance etc)
    - Beware of mouse wranglers (AKA King Kong Syndrome)...
- More handling →
  - Lower body wt in DIO models (length of study?)
  - Improved glucose tolerance in DIO models
  - Less stress response to handling in long studies

One word about Chemistry reference ranges (intervals):

LIES.

- Lies, Damn Lies, and Reference Intervals (or Hysterical Control Values for Clinical Pathology Data).
- Concurrent relevant controls are critical.

Clinical Chemistry: False Phenotypes e.g. & especially: Hemolysis

- Causes
  - Mouse wrangling
    - Specimen abuse
    - Small needle
    - Suction
    - Evaporation
  - Real phenotype?
    - Not usually
      - Fragile RBC's
      - Hemolytic syndrome
- Effects - Phenotypes?
  - Or just ARTIFACTS?
- RBC
- AST
- LDH
- K+, P
- Glucose
More false phenotypes
A sad story of sorry specimens ...

- Hemolyzed serum samples
  - LDH & AST
  - EDTA Blood
  - separated 1-2hr later
  - glucose
  - Plasma not hemolyzed
  - WNL LDH AST
  - BUT NO ALP activity
    - EDTA chelated Mg Zn

More false phenotypes
A sad story of sorry specimens ...

More false phenotypes
A sad story of sorry specimens ...

Interpreting Clinical Pathology
Results

Multiplex options:

- $250 / 80ul Serum? or Plasma?
- Clotting factors?
- Future of clinical chemistry?
- Radioimmunoassays (RIA): ACTH, corticosterone, cortisol, c-peptide, ghrelin, glucagon, growth hormone, insulin, leptin, PYY, testosterone, T3 and T4
- High Performance Liquid Chromatography (HPLC): amino acid, catecholamines (epinephrine & norepinephrine), creatinine and purine nucleotides
- Luminex multiplex: includes human and mouse adipokine, cytokine/chemokine, metabolic hormones, IGF, immunology/inflammation, and pituitary panels

https://www.vumc.org/hormone/
Acute Phase Response

- Stress, trauma, Infection, Inflammation, Neoplasia
  ◦ LPS, opsonins, neural signals
- Local response → proinflammatory cytokines
  ◦ IL1, IL6, TNFα; Neutrophil activation
- Liver hepatocytes → APP etc proteins
- Acute Phase Response proteins (APP)
  ◦ Leukocytosis; Complement Activation, Protease inhibition, clotting opsonization,

Major & Moderate Acute Phase Proteins (APP)

<table>
<thead>
<tr>
<th>sp</th>
<th>Major &gt; 10x</th>
<th>Moderate 1-10x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>C-reactive protein, serum amyloid A</td>
<td>a1-acid glycoprotein, fibrinogen, haptoglobin</td>
</tr>
<tr>
<td>Mouse</td>
<td>haptoglobin, serum amyloid A, serum amyloid P</td>
<td>C-reactive protein, fibrinogen</td>
</tr>
<tr>
<td>Rat</td>
<td>a1-acid glycoprotein, a2-macroglobulin</td>
<td>C-reactive protein, fibrinogen, haptoglobin</td>
</tr>
<tr>
<td>Rabbit</td>
<td>haptoglobin, serum amyloid A</td>
<td>a1-acid glycoprotein, C-reactive protein, fibrinogen, fibrinogen</td>
</tr>
<tr>
<td>NHP</td>
<td>C-reactive protein,</td>
<td>a1-macroglobulin, fibrinogen, Serum amyloid A</td>
</tr>
</tbody>
</table>

- Reviewed in Cray & al. 2009

Mouse Urinalysis

- Volume is a problem....
- ‘Opportunistic’ usually < 50ul
- 24hr with Metabolic cage
  - Contamination
    - Feces, bacteria, food
  - Evaporation

Urine Collection

- Small volumes
  - Opportunistic, am vs pm, fasting....
  - Mice with no food don’t drink either....
  - Parafilm, foil, mirror, microtiter plates
  - WAIT after transporting...
- 24 hr collections
  - Special caging
  - Contamination
  - Evaporation

Urinalysis

- Proteinuria is normal
  - α & β u-globulin
  - Prealbumins (mouse urinary proteins)

Refractometers

- Veterinary → 1.080
- Mice SG > 1.040
- Human SG < 1.030
- Water SG ~ 1.010
- 50ul!

Urinalysis Strips / Dipsticks

- ~10ul per test spot
- Qualitative
- Validated for
  - Specific gravity?
  - WBC?
  - Glucose?
  - Protein?
  - Blood?

Practical Clin Path (Phenotyping) Conclusions

1. Minimally invasive and relatively inexpensive tests can provide valuable and valid data.
   - Design studies to take advantage of these
2. (Mis) handling of animals or specimens can lead to false phenotypes
   - Learn about the tests & complications 1st
   - Standardize protocols - they must be repeatable.
3. Concurrent Relevant controls are essential.
QUIZ: which of these Test Conditions can influence clinical pathology results/phenotypes?

1. Time of day, most mice are nocturnal with circadian rhythms that influence times of eating and activity;
2. Fasting or water withholding periods;
3. Room temperature and/or temps in/on test equipment;
4. Stress of handling, transportation or cage changing;
5. Anesthetic effects;
6. Anatomic site for blood withdrawal (central vs peripheral samples);
7. Tube type, anticoagulant, dry (lyophilized) vs wet (dilution effect);
8. Plasma vs serum, time to separation, storage, freezing, thawing;
9. Assay methods and equipment (e.g. wet, dry, open, closed chemistry systems)

Resources

- IMPRESS European Mouse Phenotyping Resource of Standardized Screens
  http://www.mousephenotype.org/impress
- MMPC Mouse Metabolic Phenotyping Centers (sponsored by NIDDK, NHLBI)
  http://www.mmpc.org/
- MPD Mouse Phenome Database with data & protocols
  http://phenome.jax.org/
- Nomenclature of mice and genes
  http://www.informatics.jax.org/mgihome/nomenclature/index.shtml

References

- Russell, J. (2014) Comparative Performance of IDEXX Procyte Dx Hematology Analyzer and the Siemens ADVIA 2120 Hematology System Based on CBC Analysis in Mice, Rats and Guinea Pigs. IDEXX Biossearch.

Metabolic Phenotyping

- Fat mice ....
  - Pretty obvious - they weigh more ...
  - Body weight data, aka ‘growth’ curves are about the cheapest data you can get ...
  - Body composition etc metabotyping $$$

Metabo-typing

- Body Composition: QNMR
  - Short term restraint (in tube)
    - 47 sec acquisition

Metabo-typing

- Body Composition: DEXA
  - Dual-energy x-ray absorptiometry
    - Sedation / anesthesia
      - 2 min acquisition
    - Reproducibility ?
      - Phantom control
    - Total body density/composition
    - Bone mineral density
      → estimate % fat

More Metabo-typing Options

- C:
  - n = 5-10 M + 5-10 F
  - (m/m, +/-; Tg/+, +/-)
1. Growth curve from 3wo → Weekly body wt
2. 4wo → biweekly
  - blood gluc, lipid, BP
3. 8wo → Monthly
  - Echo/ECG + Body Comp (DEXA)
  - Diet challenge
  - Consumption (Metab cage) + growth curve from 3do (2/wk)
  - Gluc tolerance
  - MAP 60
  - Calorimetry etc (B----)
  - Pathology
- J:
  - n = 5-10 M + 5-10 F
  - (m/m, +/-; Tg/+, +/-; Rx)
1. Growth curve from 1 → Weekly body wt
2. 4wo → weekly
  - Glucose - Glucometer glucose
3. Weekly food/H2O consump
4. 8wo → Monthly
  - Echo/ECG + Body Comp (QNMR)
  - Diet challenge
  - Dosing
  - Gluc tolerance
  - Calorimetry, activity etc (C----)
  - Terminal chemistry
  - Pathology

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- Nomenclature of mice and genes
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Where to find Phenotype Data and Protocols

♦ Data
- IMPC - search by gene, phenotype, etc
- http://www.mousephenotype.org

♦ Protocols
- IMPReSS (International Mouse Phenotyping Resource of Standardised Screens)
- formerly EMPReSS
- http://www.mousephenotype.org/impress
- DATA, Protocols, references

Hologic (Faxitron)
- Table top digital imaging and pre-clinical DEXA systems
- Highest resolution (<10µm)
- Largest field of view in the market.
- Vs microCT or 3D imaging
  - less expensive,
  - lower doses,
  - faster results.
  - no other shielding required.
  - ~$130,000.
  - ~10%/an serv contract
  - Christmas is coming 😊 ...

Faxitron MX20
- Hi resolution 2d flat film radiography

Faxitron MX20
- Hi resolution 2d flat film radiography