Galveston Porphyria Laboratory

The University of Texas Medical Branch

Galveston, Texas

Instructions for Collecting, Processing and Shipping Samples for Porphyria Testing

This packet contains the following:

- **Instructions for collecting, processing and shipping samples**
- **Order form for testing**
- **A primer on laboratory testing for porphyrias**

Updated 9 June 2021

Instructions for Collecting, Processing and Shipping Samples

I. Sample Collection and Processing

GENERAL INSTRUCTIONS

1. Types of samples used for porphyria testing (For choice of tests, see the attached Primer):
    a. Urine – spot (random or single void) urine sample is recommended with no preservative. A first-void sample on arising in the morning is preferred.
        i. Creatinine is measured on all urine samples for "normalization" of the results. The amount of creatinine excreted every day is quite constant because it reflects muscle mass. Most adults excrete 1-2 grams of creatinine daily in urine. Expressing results per gram of creatinine corrects for variation in the hydration state of the patient over time. The sample should be light protected (e.g. by wrapping the container in aluminum foil) and immediately refrigerated or frozen.
        ii. A 24-hour collection is also suitable, but 5 gm of sodium carbonate (not sodium bicarbonate) should be added to the container before starting the collection, the container should be refrigerated and protected from light during the collection (e.g. by using a dark-colored plastic collection bottle). The total volume must be measured in a single container and only a portion (an "aliquot") sent to the laboratory for testing. Detailed instructions on how to properly collect a 24-hour urine must be given verbally to the patient along with the container containing the preservative.

        Sample labeling and light protection: see below.
    b. Whole blood – for erythrocyte tests. Test results are expressed per mL of erythrocytes, based on the hematocrit of the sample. Samples are collected in a green top (heparin anticoagulant) tube. A lavender top (EDTA anticoagulant) tube may also be suitable.
    c. Plasma – some of the whole blood sample is centrifuged, and plasma removed and frozen.
    d. Serum – whole blood in a red top tube (no anticoagulant) is allowed to clot, centrifuged after the clot has contracted, and the serum removed and frozen.
    e. Stool (feces) – spot sample – if possible, should be at least 20-40 grams or 2 tablespoons or about the size of a golf ball.
2. Protect samples from light and keep refrigerated during collection and processing. Wrapping sample containers in foil is a convenient and effective way to protect from light. Some light exposure is necessary during sample collection and processing, but should be minimized.
3. All samples must be labelled with the patient's name, date of birth, date the sample was obtained, and the type of sample (e.g. spot urine, plasma, etc). Labels must be placed on containers before wrapping in foil or freezing, because cold containers may not retain labels or marking.
4. Refrigerate or freeze samples soon after collection and processing.
5. Plastic rather than glass containers should be used for freezing and shipping samples to avoid breakage. Containers should be only 2/3 full, if samples are to be frozen, and tops should fit tightly to prevent sample leaking or evaporation.

URINE SAMPLES

For measurement of urine ALA, PBG, porphyrins and creatinine.

Proper labeling of all samples is essential

- All sample tubes and containers must be labeled ***before sample collection*, *before making secondary aliquots and before refrigerating or freezing***
- Labels adhere poorly and lettering may fade if applied to wet or cold containers
- Labels must be applied to containers before wrapping with aluminum foil. Applying a second label on the aluminum foil is optional.

1. A spot (random or single void) urine, preferably collected on arising the morning, is recommended (see above). No preservative is used.
    a. If a 24-hour sample is collected, add 5 grams sodium carbonate as a preservative to the empty container. (If this preservative is not available call us and we will send it by mail. If unable to wait for Na carbonate, it is better to use no preservative rather than an acid preservative or any other alternative preservative.) *Detailed instructions on how to collect a 24-hour urine is provided separately and must be followed closely.* Keep the sample refrigerated and shielded from light (e.g. use a dark brown container) during and after the collection. Sample processing: After the collection is complete, the sample is mixed thoroughly, and the total volume measured in a single container. If some solid material has precipitated, it should be mixed also with the whole sample. A 20-50 mL aliquot of the mixed sample is removed to an unbreakable plastic container, tightly screw-capped, and refrigerated or frozen. A 50 mL tube with screw cap is preferred. (The container for the urine aliquot should not be filled more than 2/3 full, or it will leak or break during freezing.) Record the total volume on the form provided and on the label of the aliquot.
2. Label the spot urine sample or the aliquot of the 24-hour urine with "Urine", the patient's name, date of birth, the collection date, and the 24-hour urine volume (if applicable). It is not necessary to record the volume of a spot sample.
3. Wrap the sample with aluminum foil after it is labelled. (The label should never be on the foil.) Note that labels are best applied at room temperature rather than after the sample is refrigerated or frozen.

WHOLE BLOOD SAMPLES. Note that, depending on the testing requested, whole blood samples may be shipped either:

- **Unprocessed samples sent at ambient temperature or refrigerated.** This is recommended for erythrocyte protoporphyrin/total porphyrins and plasma porphyrins, if samples can be shipped overnight to arrive when the laboratory is open (usually Mondays through Fridays, unless closed for a holiday or a weekend). It is not necessary to provide a hematocrit report if samples are shipped unfrozen.
- **Processed samples sent frozen on dry ice.** A whole blood frozen lysate and a frozen plasma sample are both sent, and these are suitable for all tests on erythrocytes and plasma. This is the default method but requires attention to proper processing and shipping. Also, a hematocrit must be done on the same sample by the site and reported to us, or the site should provide a hematocrit done separately within approximately one week.

WHOLE BLOOD SAMPLES – SHIPPED AT AMBIENT TEMPERATURE OR REFRIGERATED

This is suitable for measurement of plasma porphyrins, erythrocyte protoporphyrin and erythrocyte porphobilinogen deaminase.

1. Draw blood into a heparinized (green top) tube. EDTA (purple top) tube may be suitable – check with the lab.
2. Label tube with "Whole Blood", the patient's name, date of birth, and the collection date.
3. Ship the sample at ambient temperature, or with a refrigerant pack (blue ice). Do not process or freeze the sample. *If samples are in glass tubes, extra care must be taken to pack samples well and avoid breakage.*

WHOLE BLOOD SAMPLES – SHIPPED FROZEN. *Do not freeze blood or ship frozen blood in glass tubes.* A frozen sample is especially important for measurement of erythrocyte uroporphyrinogen decarboxylase, which has limited stability when refrigerated or at ambient temperature. Other erythrocyte assays can also be done using frozen samples.

1. Draw blood into a heparinized (green top) tube. EDTA (purple top) tube may be suitable – check with the lab. Mix well, but gently.
2. Obtain a hematocrit result (e.g. by sending a separate sample to a clinical laboratory). Record the result on the form or send us the result separately when it becomes available. (A recent – within about one week – hematocrit value can be provided, if the patient's clinical condition has not changed in the interim.
3. Transfer 0.5-1.0 ml of well-mixed whole blood into a small plastic tube with a stopper. The tube should be only up to 2/3 full, to allow for expansion. It is important to then close the stopper tightly.
    a. Label tube "Whole Blood" and with the patient's name, date of birth, and the collection date. Labelling should be done before freezing.
    b. Freeze immediately, so red cells and plasma do not separate before freezing. Plastic tube is placed on its side in a -20^{o}C freezer or on dry ice, or flash-freeze by dropping it into acetone-dry ice.

PLASMA SAMPLES

1. Obtain heparinized whole blood (may in a separate tube or the remainder of the blood used for assays requiring whole blood, as described above).
2. Centrifuge to separate the plasma.
3. Transfer 0.5-1.0 ml plasma to a small plastic tube with a stopper, label as "Plasma", and with the patient's name, date of birth, and the collection date and then freeze. Tube should be only 2/3 filled to compensate for expansion during freezing. Labelling should be done before freezing. It is important to close the stopper tightly before freezing.

SERUM SAMPLES

For measurement of serum porphobilinogen (plasma may be used instead, but if so, it should be recorded on the form and the sample as "Plasma").

1. Draw blood into a red top tube (no anticoagulant, allow clot to form and retract, centrifuge and save at least 2.0 ml serum in a plastic tube.

2. Label as "Serum", and with the patient's name, date of birth, the collection date and then freeze. For freezing, follow the instructions as above for plasma.

STOOL (FECES) SAMPLES

For measurement of stool porphyrins:

1. Obtain a random stool sample (at least 20-40 grams or 2 tablespoons or about the size of a golf ball).
2. Freeze the sample in an unbreakable, plastic, air-tight container. The container should be placed in a plastic bag and can be refrigerated initially for up the 24 hours, and should then be frozen at -20^{o} or -80 ^{o} C before shipping. Samples are stable at -20^{o} for at least one month, or longer at -80 ^{o} C
3. Label as "Stool" or “Feces”, and with the patient's name, date of birth and the collection date. Labeling should be done before refrigerating or freezing to assure the label is adherent and the written information is legible.

II. Instructions for Packing and Shipping Samples

1. Fill out the attached Request Form and provide the needed information for all samples and specify which tests are requested. Provide clinical information on the form, if available. Refer to the attached Primer, or consult the laboratory by phone, email or letter if there are questions about which tests are needed.

2. Note that we are unable to provide kits or containers, to pay for shipping, or to bill insurance.

3. Ship prepaid by an overnight air transportation company that will deliver directly to The University of Texas Medical Branch.

3. Pack samples to be shipped either:

- At ambient temperature or with a refrigerant pack – only for whole blood, as noted above. If samples are to be shipped in glass tubes, they must be packed carefully to avoid breakage.
- With dry ice – for whole blood, plasma, serum, urine and stool samples. Pack the samples with enough dry ice to last for 3 days (usually 5-10 pounds). Use a Styrofoam insulated shipping container that is enclosed in a cardboard box. All samples should be in unbreakable plastic tubes or containers that are tightly capped prior to freezing. *Note that some plastic containers are brittle when frozen and therefore not unbreakable.* Samples sent from outside the US may require more dry ice due to longer shipment times.
- Samples in the container should be wrapped or padded to prevent damage in transit.

4. Ship to:

Galveston Porphyria Laboratory	*Write on shipping form:* **"Inside Delivery Only"**
Attn: Karl E. Anderson, M.D.	
University of Texas Medical Branch	
301 University Boulevard, BSB 4.128	Telephone: (409) 772-4661
Galveston, Texas 77555-0655	FAX: (409) 772-6287

5. **Ship early in the week and avoid arrival of samples on weekends or university holidays. If in doubt, call or send an email to confirm before shipping when the laboratory is open.**

[Patient Label (if available)]

Test Request Form

Galveston Porphyria Laboratory

University of Texas Medical Branch
301 University Boulevard, BSB 4.128
Galveston, Texas 77555-0655
Phone: 409-772-4661 Fax: 409-772-6287
CLIA # 45D2204212 COLA ID: 30751
Write on shipping form: "***Inside Delivery Only***"

Name: _____

Age: _____ DOB: _____ Sex: _____

Diagnosis _____

Hematocrit: _____ (Date _____)
Hematocrit is required when erythrocyte tests are requested, because these tests are done on whole blood and the hematocrit is used to calculate results.

Reticulocytes: _____ (%) (Date _____)
Reticulocyte count is optional, but is suggested for the erythrocyte tests, because young erythrocytes can increase the results.

Clinical features: *(to facilitate interpretation of results)*

❑ Abdominal pain ❑ Other pain

❑ Peripheral neuropathy

❑ Skin Blisters ❑ Other skin lesions

❑ Other features: _____

Results are reported with a combined interpretation that includes consideration of any clinical information provided.

❑ Hematocrit and/or reticulocyte results will be sent later.

URINE (3 tests bundled):	Collection Date:	CPT Codes	Charges
❑ **δ–Aminolevulinic acid (ALA)**	**Time:**_____	82135	$85 for
❑ ***Porphobilinogen (PBG)**		84110	3 tests
❑ ***†Total Porphyrins** (Reflex: HPLC fractionation if total increased)		84120 (same with HPLC)	(+$80 for HPLC)
❑ **Spot/random urine** ***or*** ❑ **24 hour urine (total volume _____mL)**			
PLASMA (2 tests bundled):	**Collection Date: Time:**		
❑ **†Total Porphyrins & Fluorescence Scan**		84311	$75
FECES:	**Collection Date: Time:**		
❑ **Total Porphyrins** (Reflex: fractionation by HPLC if increased)		84126 (same with HPLC)	$75 (+$80)
SERUM:	**Collection Date: Time:**		
❑ **Porphobilinogen (PBG) – *** only for patients with renal failure		84110	$80
ERYTHROCYTE (Whole Blood):	**Collection Date: Time:**		
❑ **‡Protoporphyrin** (total porphyrins, expressed as protoporphyrin) Reflex: metal-free & zinc protoporphyrin if increased)		84202	$75
❑ **Porphobilinogen deaminase (PBGD)**		82657	$85
❑ **Uroporphyrinogen decarboxylase (UROD)**		84999	$170

Name and address of physician, laboratory or other individual where results should be sent:

Physician Signature: _____

Name & address to whom invoice should be sent (We do not bill insurance directly):

See attached instructions regarding sample preparation and shipment.

Recommended for initial screening for *acute porphyrias, †blistering cutaneous porphyrias, or ‡nonblistering cutaneous porphyrias (protoporphyrias). For further information see "*Primer on Laboratory Testing for Porphyria*", or call 409-772-4661.

Tax deductible donations to UTMB to support porphyria research may be sent to the address above.

A Primer on Laboratory Testing for Porphyrias

What causes porphyrias? Porphyrias result from altered activity of the 8 enzymes in the heme biosynthetic pathway, which is shown in **Figure 1**. Broadly speaking there are 8 types of porphyria, each due to an abnormality in one of these enzymes. Inherited loss of function mutations that result in enzyme deficiencies are responsible for most porphyrias. But one type of porphyria results from gain of function mutations, and another results from an enzyme inhibitor that is generated in the liver even in the absence of a mutation (1).

What are enzymes? Enzymes are proteins made by cells in the body for very specific purposes, such as combining two molecules (enzyme substrates) to make a third molecule (the enzyme product). Enzymes *catalyze* the reaction of its *substrate(s)* to form a *product*. Enzyme substrates are referred to as the *precursors* of the product. A **metabolic pathway** consists of multiple enzymes and their substrates and products, which lead to synthesis of an important substance, such as heme. Another pathway leads to breakdown of heme (to bilirubin and iron).

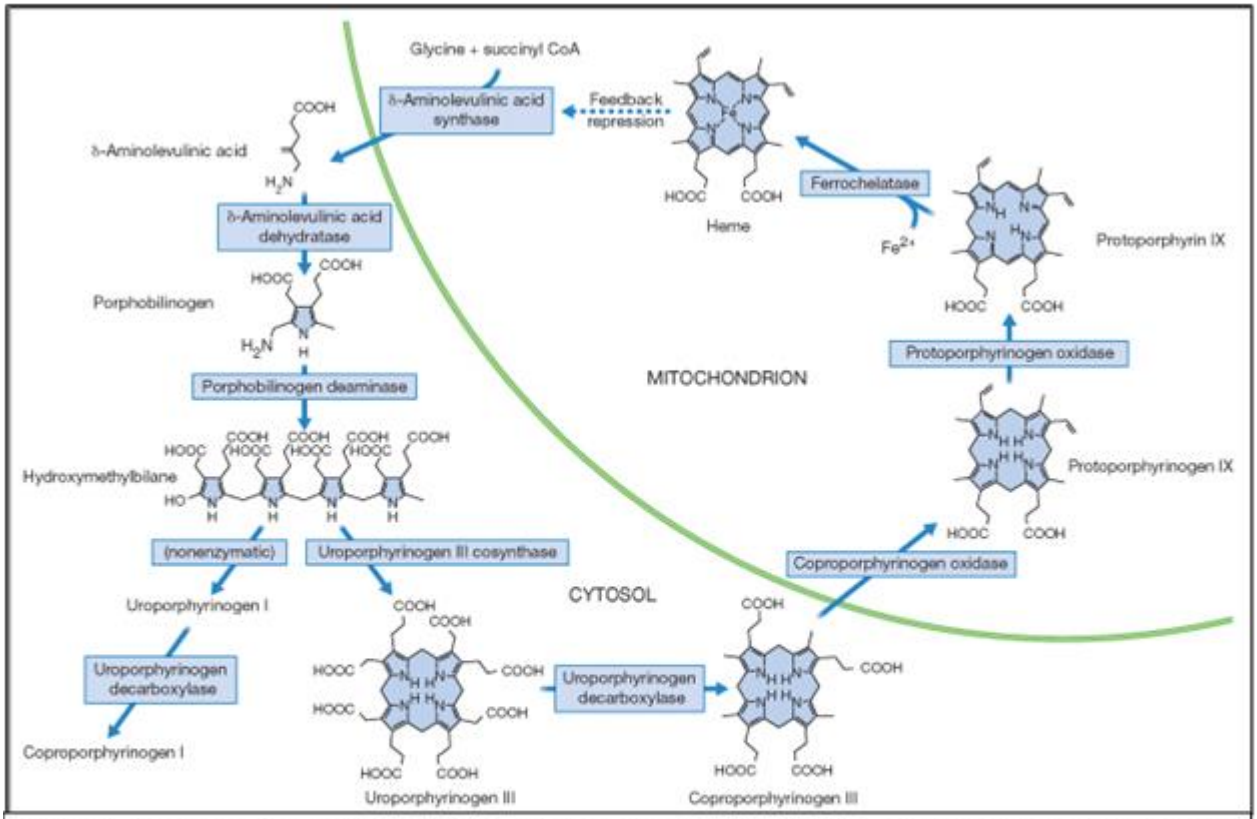

Figure 1. The heme biosynthetic pathway. Intermediates include an amino acid (delta-aminolevulinic acid – ALA), a pyrrole (porphobilinogen – PBG), a linear tetrapyrrole (hydroxymethylbilane) and a series of porphyrins (cyclic tetrapyrroles), of which only the asymmetric type III isomers lead to heme. Except for protoporphyrin IX, the porphyrin intermediates are in their reduced forms (porphyrinogens). Starting with uroporphyrinogen III, 6 of 8 carboxyl groups are removed to form protoporphyrinogen. The latter is oxidized to protoporphyrin, followed by insertion of iron (Fe) to make heme.

What is heme? Heme is large porphyrin molecule with an iron atom at the center. Its structure is shown as the end product of the pathway in **Figure 1**. Heme is a component of many essential hemoproteins (proteins that require heme to function) in the body (e.g. hemoglobin, myoglobin, cytochromes and many others). Heme cannot be absorbed intact from outside the body, so it must be made by all cells at some

time during their life cycles. The largest amounts of heme are made in the bone marrow (mostly for hemoglobin) and liver (mostly for cytochrome P450 enzymes). Hemoglobin synthesis is completed in the marrow by erythrocyte precursor cells. Mature erythrocytes that circulate in blood no longer make heme. Their main function is to transport oxygen, which is bound to hemoglobin as the cells pass through the lungs, and then to release oxygen in other tissues. Cytochromes P450 are enzymes that use oxygen to metabolize many chemicals, hormones and drugs, and are most abundant in the liver.

The pathway to make heme is comprised of eight enzymes and their substrates and products. **Figure** 1 shows the eight sequential reactions catalyzed by these enzymes and the chemical structures of the pathway intermediates. All substrates and products in the pathway, including some that are porphyrins, are referred to as "heme precursors". Delta-aminolevulinic acid (ALA) and porphobilinogen (PBG) are formed early in the pathway by the first and second pathway enzymes, respectively, and are also known as "porphyrin precursors".

Within cells, all intermediates are colorless and nonfluorescent, with the exception of the last intermediate, protoporphyrin IX. When ALA accumulates, it may be taken up by other cells and further metabolized to porphyrins and heme. When PBG accumulates, it may form porphyrins (which are red and fluorescent) or a degradation product known as porphobilin (which is brownish). When they accumulate outside cells, the porphyrinogen intermediates in the pathway become autooxidized to their corresponding red and fluorescent porphyrins. Therefore, porphyrins and PBG derived from heme precursors account for reddish or brownish urine in many types of porphyria.

What are I and III isomers? PBG is an asymmetric molecule because it has two different carboxyl side chains. When 4 PBGs are assembled to form porphyrinogens, the different products may have the same number of atoms but with structures that differ in their symmetry, depending on the orientation of the side chains. These are referred to as different *isomers*. There are many possible isomers, but formation of the III isomer of uroporphyrinogen is essential for making heme. The third enzyme in the pathway forms hydroxymethylbilane (HMB, a transient, unstable linear tetrapyrrole), with all 4 PBGs having the same orientation (**Figure 1**). The next step, catalyzed by the fourth enzyme, forms uroporphyrinogen III, which is asymmetrical, and to do this one of the pyrroles is flipped over before closing the porphyrinogen ring. Otherwise, HMB cyclizes spontaneously without flipping one of the PBGs, to form uroporphyrinogen I, which a symmetrical molecule. Although the fifth enzyme accepts both uroporphyrinogen I and III, forming coproporphyrinogen I and III, respectively, the sixth enzyme accepts only coproporphyrinogen III, so coproporphyrinogen I cannot be a heme precursor. Relative amounts of porphyrin isomers I and III in urine and feces are sometimes diagnostically important.

How are different heme precursors excreted? Porphyrinogens are mostly oxidized to porphyrins before excretion. ALA, PBG and highly carboxylated porphyrins (i.e. with 6-8 carboxyl side chains) are water soluble and excreted mostly in urine. Those with fewer carboxyl side chains are less soluble and excrete in bile and feces. Protoporphyrin, with only 2 carboxyl side chains, is completely insoluble in water, and is totally excreted in bile by the liver, and appears in feces rather than urine.

Can the enzymes that are altered in porphyrias be measured? Some of the pathway enzymes are found in mitochondria within cells and others are in the cytoplasm (**Figure 1**). Activity of the latter enzymes can be measured in erythrocytes. But mature erythrocytes have lost their mitochondria, so the mitochondrial enzymes (the first and final 3 enzymes, see **Figure 1)** are more difficult to measure and must be measured in other types of cells. The more recent development of DNA testing, which can identify actual mutations, has made measuring enzymes less important.

Is DNA testing definitive? DNA testing has become standard of care in patients with porphyria (2). It confirms the diagnosis in an "index case" and enables accurate identification of family members who have inherited the same mutation. They can then be counseled to avoid triggers that might cause symptoms. But DNA results must be interpreted with caution, and biochemical test results remain important. For example, porphyrias are often latent, so finding a mutation does not explain symptoms

unless elevations in heme precursors are also demonstrated. Also, some variants in the genetic code do not cause disease or at present are of unknown significance (3). Therefore, before family members are tested, it is best to establish the biochemical abnormalities and responsible mutation(s) in the index case.

How are porphyria classified, and do they have much in common? Porphyrias are classified as *hepatic* or *erythropoietic* depending on whether the accumulation of intermediates first occurs in the liver or marrow.

They are also categorized into 3 broad types by symptoms as: ***1) acute, 2) blistering cutaneous***, or ***3) nonblistering cutaneous***. The 3 most common porphyrias happen to exemplify these categories and *share no common features* and as shown in **Table 1 (first column)**. Other less common porphyrias share features with the 3 most common types (1).

Are all porphyrias tested for in the same way? No single test can be ordered to detect or exclude all porphyrias. Ordering a test that does not exist, such as "porphyria screen" or "porphyrin profile" can lead to confusion and incorrect testing.

Recommended first-line testing for each of these 3 most common types of porphyria is shown in **Table 1 (second column).** This first-line testing is limited, making it cost-effective to consider porphyrias more often in the differential diagnosis. This approach is also sensitive, so when first line testing is negative, no further testing for porphyrias as the cause of symptoms is needed. If abnormal, more extensive second line testing should follow. Less common porphyrias (**Table 1, third column**) are also detected (or excluded) by this first-line testing strategy (4-6).

Table 1. The 3 most common porphyrias, their principal presenting features (in italics), recommended first line testing when suspected, and the other less common porphyrias with shared presenting features that will be detected by the same first line testing.

The 3 most common porphyrias and their *principal clinical features*	**Recommended first line testing**	**Less common porphyrias with the same or similar features, which are differentiated by second-line testing**
1. Porphyria cutanea tarda (PCT) *Chronic blistering photosensitivity*	**Urine or plasma total porphyrins**	• Congenital erythropoietic porphyria (CEP) • Hepatoerythropoietic porphyria • Hereditary coproporphyria (HCP) • Variegate porphyria (VP)
2. Acute intermittent porphyria (AIP) *Abdominal pain, neurological symptoms*	**Urine porphobilinogen and total porphyrins**	• HCP • VP • Delta-aminolevulinic acid dehydratase porphyria (ADP)
3. Erythropoietic protoporphyria (EPP) *Acute nonblistering photosensitivity*	**Erythrocyte total porphyrins/protoporphyrin**	• X-linked protoporphyria (XLP)

Can a diagnosis of porphyria be established based only on symptoms? No, because the symptoms of porphyria (e.g. abdominal pain, other neurological symptoms, skin blistering, painful photosensitivity) are very nonspecific. Therefore, porphyria can be suspected based on nonspecific symptoms, but a diagnosis must be established by laboratory testing.

When should one test for porphyrias? Porphyria should be part of the differential diagnosis of symptoms that remain unexplained after initial testing for more common conditions. A high level of suspicion should not be required. These are rare diseases with nonspecific symptoms, and are often diagnosed when the level of suspicion before testing is not very high. For example, **Table 2** lists some clinical scenarios associated with severe, nonspecific symptoms and signs that should prompt testing for acute porphyrias.

Is testing readily available and effective? Porphyrias are readily ruled in or out by widely available, sensitive and cost-effective biochemical tests, especially when symptoms are present. Available testing

includes measurement of excess porphyrin precursors (ALA and PBG) and porphyrins in plasma, erythrocytes, urine and feces. As will be discussed below, only a first-line test rather than a battery of tests is required to exclude an active porphyria as a cause concurrent unexplained symptoms.

Table 2. Symptoms, signs and laboratory findings that should prompt testing for acute porphyrias.
Abdominal pain, unexplained after an initial standard workup
Peripheral neuropathy, especially acute and progressive, including Guillain Barre syndrome
Hyponatremia
Red or dark urine, without increased red blood cells
Hallucinations, or other acute CNS symptoms

Some diagnostic strategies to avoid. 1) *Refer the patient to a specialist for initial testing*. First line testing for acute porphyrias is most sensitive and most likely to be positive at the time of symptoms. Testing for elevated PBG and porphyrins in urine is widely available and does not require specialist referral. Referral is not necessary unless first line testing is positive. 2) *Perform all available tests when porphyria suspected.* This is unwise because some tests lack specificity, and their overuse can lead to confusion and misdiagnoses (7, 8). 3) *Order genetic testing to begin with when porphyria is suspected*. Rarely, a pathogenic mutation may be present but not be identified, particularly by sequencing. An identified variant might not be diagnostic because i) known pathogenic variants are often latent and do not explain symptoms unless accompanied by elevations in porphyrin precursors or porphyrins, and ii) a benign variants or "variant of unknown significance" (VUS) do not support a diagnosis of porphyria (3).

What is the recommended approach to screening for porphyrias? When porphyria is first suspected, a first line screening test should be chosen based on the presenting symptoms of the 3 most common porphyrias (**Table 1**). All physicians are likely to encounter patients with these porphyrias at some time in their careers. Screening results will usually be negative, which will exclude porphyria and thereby contribute to the patient's workup in a cost-effective manner. Costs of further testing for porphyria is needed only when first line testing is abnormal.

1. Testing for *acute porphyrias as causes of neurovisceral manifestations*. Some clinical scenarios associated with the 4 acute porphyrias were listed in **Table 2**. These are referred to as acute hepatic porphyrias (AHP), because excess porphyrin precursors originate in the liver in AIP, HCP and VP, which are autosomal dominant inherited diseases with variable penetrance. However, an erythropoietic component may contribute in ADP, which is autosomal recessive, and in homozygous cases of AIP, HCP and VP.

First-line testing for acute porphyrias relies heavily on measurement of PBG, because substantial elevation of PBG is both sensitive and specific for the 3 most common acute porphyrias – AIP, HCP and VP. PBG should be measured by column chromatography or mass spectrometry (12, 13). Qualitative testing for elevated PBG (e.g. Watson-Schwartz or Hoesch tests) is no longer recommended, but may still be used for rapid testing because unfortunately, a reliable kit that tested semiquantitatively for elevation of urine PBG (14) is no longer available.

Measurement of PBG and total porphyrins is recommended for first line testing, with reporting of results as rapidly as can be arranged with the testing laboratory. Measurement of delta-aminolevulinic acid (ALA) is not necessary for screening, since ALA is less elevated in AIP, HCP and VP, and conditions that elevate ALA (but not PBG), such as ADP and lead poisoning, also increase urine porphyrins.

Urine total porphyrins are measured as part of first line testing because PBG may be less elevated and fall more rapidly after an attack in HCP and VP than in AIP. Measuring urine porphyrins will also detect symptomatic cases of ADP, which elevates urine ALA and coproporphyrin III. ALA dehydratase is also deficient, and urine ALA and coproporphyrin III increased, in lead poisoning and hereditary tyrosinemia type I. Therefore, normal PBG and total porphyrins effectively excludes all 4 AHPs, as well as these additional conditions associated with decreased ALAD activity and porphyria-like symptoms.

A spot (single void) sample with no preservative (rather than a 24-hour collection) is recommended for measurement of urine PBG and porphyrins. The spot sample should be promptly light protected and refrigerated or frozen until PBG and porphyrins are measured. Results may be reported initially as concentrations (e.g. mg/L), but the final result should be normalized to creatinine, to correct for the degree of hydration of the patient being tested.

Requiring a 24-hour urine collection can considerably delay diagnosis or exclusion of acute porphyria. Furthermore, urinary PBG excretion may decrease considerably (especially in HCP and VP) if there is a delay of in collecting a 24-hour urine, or if the patient is treated with intravenous hemin. Moreover, collection of a 24-hour urine is difficult and inaccurate for many patients. If a 24-hour collection is collected, sodium carbonate (5 grams, added to the urine bottle prior to collection) is recommended for urine specimens intended for measurement of ALA, PBG and porphyrins. Some laboratories require that acid be added to 24-hour containers for collection of urine for ALA determination, because this substance is more stable in acid. But acid conditions enhance degradation of PBG. Therefore, rather than 2 separate collections, it is preferable to use either sodium carbonate or no preservative for measurement of ALA, PBG and porphyrins. The container should be refrigerated and protected from light during the 24-hour collection.

PBG can also be measured in serum or plasma, but their concentrations are lower than in urine if renal function is not impaired. Therefore, measuring serum PBG is important primarily in patients with renal impairment.

The cost of more extensive second line laboratory testing is justified if urine PBG and/or porphyrins are elevated, and will determine whether or not PBG and/or porphyrin elevation is due to porphyria, and if so the type of porphyria. A substantial elevation in PBG is specific for AIP, HCP or VP, but slight elevations may not be diagnostic, especially if a sensitive method such as mass spectroscopy is used. Even substantial elevations in urine porphyrins are not specific for porphyria, and other causes need to be considered especially is urine porphyrin elevation is an isolated finding. At the stage of second-line testing, a physician and laboratory with experience in testing for and clinical management of these disorders should be consulted (15, 16).

The upper limits of normal (ULNs) for ALA and PBG vary greatly between laboratories, in part due to differences in methodology. The ULNs are considerably lower, for example, using mass spectrometry than with column chromatographic and colorimetric methods. Therefore, interpretation of results based on fold elevation above an ULN can be misleading, and *actual levels normalized to creatinine, which are expected to be markedly elevated in cases of active porphyria, should be relied upon for interpretation*.

Increased in urinary porphyrins, especially coproporphyrin, are seen in many medical conditions, including liver and bone marrow diseases. Nonspecific increases are usually slight or moderate, but marked elevations are sometimes seen in liver diseases and other conditions. *Active porphyrias are distinguished by specific patterns of elevation of porphyrin precursors and/or porphyrins, as assessed by second line testing*.

Fecal porphyrin determinations. Fecal porphyrins can be increased in several types of porphyrias. They are most strikingly increased and diagnostically useful in HCP (mostly coproporphyrin III) and VP (mostly coproporphyrin III and protoporphyrin). It is best to use a spot fecal sample, and express results per gram dry weight. A 24-hour collection of feces is not a meaningful timed sample, given wide variations in fecal flow. Substances in the diet may also complicate the results and their interpretation. Striking elevations are helpful for diagnosis or exclusion of certain porphyrias, and especially HCP and VP. An elevated ratio of coproporphyrin III to coproporphyrin I may identify latent cases of HCP and VP even if total fecal porphyrins are not elevated. As noted below, plasma fluorescence scanning is also especially useful for diagnosis of VP (5, 9).

2. Testing for *porphyria cutanea tarda* and other porphyrias causing chronic, blistering photosensitivity. Porphyrins are elevated in plasma and urine whenever blistering skin lesions are due to porphyria. Therefore, total porphyrins in urine or plasma should be measured as first line testing for PCT and other porphyrias with blistering cutaneous manifestations. Normal results will exclude these conditions.

For urine testing, spot (single void) urine samples are preferred. Results may initially be reported as concentrations (i.e. per L), but final results should be normalized to creatinine (i.e. expressed per g or mmol creatinine).

Porphyrins are often elevated, especially in urine, in other medical conditions. Thus, total porphyrin measurements in urine and plasma are sensitive for the presence of active porphyria, but not specific. Further testing is needed to establish that porphyrin elevation is due to porphyria, and if so, which type. Second line testing includes:

- Fractionation of urine or plasma porphyrins (see **Box**), which is important in PCT for demonstrating a predominance of highly carboxylated porphyrins (especially uroporphyrin and heptacarboxyl porphyrin). Although characteristic, this pattern is not absolutely specific for PCT.
- Plasma fluorescence scan (see next **Box**) to exclude VP.
- Measurement of erythrocyte porphyrins to exclude HEP and rare cases of mild CEP.
- Measurement of fecal porphyrins for demonstrating elevation of isocoproporphyrins, which is quite specific for PCT and HEP, and for excluding HCP, VP and CEP, which have their own distinctive patterns of individual porphyrins in feces.

Urine, plasma and fecal porphyrin fractionation. This is usually accomplished by high performance liquid chromatography (HPLC) to provide results for the following individual porphyrins (including their I and III isomers): uroporphyrin (octacarboxyl porphyrin), heptacarboxyl porphyrin, hexacarboxyl porphyrin, pentacarboxyl porphyrin, harderoporphyrin (tricarboxyl porphyrin) and protoporphyrin (dicarboxyl porphyrin). Amounts of individual porphyrins are best expressed as percent of the total, but some labs report quantities of each individually. *Importantly, slight elevations of one of more of these individual porphyrins in urine, plasma or feces are of little or no diagnostic significant unless the total porphyrin amount is increased.* Patterns of elevated porphyrins are almost always similar in plasma and urine. Fecal porphyrins represent those excreted by the liver, principally coproporphyrin, harderoporphyrin and protoporphyrin plus others derived from gut bacteria and foods.

Plasma fluorescence scanning. The wavelength of the porphyrin fluorescence peak in diluted plasma at neutral pH is useful for diagnosis (9-11). Peak wavelengths seen in different porphyrias are:
~620 nm: AIP & HCP (sometimes), PCT, HEP, CEP
~626 nm: VP
~634 nm: EPP, XLP
Most importantly, this test can rapidly differentiate VP from other acute porphyrias (e.g. AIP) and cutaneous porphyrias (especially PCT). VP presenting with skin lesions is often misdiagnosed as PCT and is sometimes as a consequence incorrectly treated. The distinctive peak in VP apparently results from binding of porphyrins to plasma proteins. It is not clear whether HPLC of plasma porphyrins provides such valuable diagnostic information in VP. Protoporphyrias also display a distinctive peak wavelength, but this is generally less essential for diagnosis.

3. Testing for *protoporphyrias (EPP and XLP)*. This requires measurement of erythrocyte total protoporphyrin, which is markedly increased in these conditions, and consists mostly of metal-free protoporphyrin. Urine porphyrins are not elevated. Plasma porphyrins are usually modestly elevated but sometimes normal. Fecal porphyrins are normal or slightly increased.

Erythrocyte porphyrins – testing considerations. In both health and disease, any porphyrins that remain in circuiting mature erythrocytes after earlier completion of hemoglobin synthesis in the marrow is protoporphyrin, which is iron-free but mostly chelated with zinc. But in the past erythrocyte protoporphyrin was known to be iron-free but mistakenly considered to the metal-free, so was described as “free erythrocyte protoporphyrin” (FEP). Erythrocyte protoporphyrin testing was offered for screening for lead poisoning, and although no longer widely recommended) is still offered by some laboratories *who still*

report levels of zinc protoporphyrin as "free erythrocyte protoporphyrin (FEP)" (17). Continued use of this traditional terminology is unfortunate and greatly impairs laboratory diagnosis of protoporphyrias (see **Box** on next page).

Erythrocyte zinc protoporphyrin is variably and sometimes markedly increased not only in lead poisoning but also in iron deficiency, anemia of chronic disease and in virtually any erythrocyte disorder. Reliable laboratories measure the total amount of porphyrins, which is usually expressed as protoporphyrin, since protoporphyrin (either zinc or metal-free) is almost always responsible for the increase (17). A rare but notable exception (see **Box**) are the increases in uroporphyrin I and coproporphyrin I in most cases of CEP.

Is a diagnosis of porphyria sometimes incorrect? An incorrect diagnosis of porphyria is commonly made in patients with symptoms due to other diseases. Therefore, in patients with a past history of porphyria it is important to review the laboratory results that were the basis for the original diagnosis (16).

Incorrect diagnoses of porphyria can occur in patients having minimal abnormalities in laboratory tests, such as small elevations in urinary porphyrins or porphyrin precursors that in fact have little or no diagnostic significance. Incorrect diagnoses are less likely *if reliance is placed on a few first-line tests in most clinical situations*, as described above.

Can porphyria by diagnosed in patients who had symptoms in the past? Acute porphyrias seldom become completely latent (such that all levels of porphyrins and porphyrin precursors become normal) within a short period of time. However, it does become more difficult to "rule out porphyria" if testing is delayed until long after symptom resolution. If it is clinically important to exclude subclinical porphyria in a patient with past suggestive symptoms, and if definitive testing was not conducted near the time of symptoms, a specialist physician and laboratory should be consulted to advise on the choice and interpretation of laboratory tests. At this stage, complete first- and second-line testing may be warranted. DNA testing is also an option and is informative if a known pathogenic mutation is found (3, 6, 16).

Erythrocyte porphyrins – features and measurement pitfalls.

- Ferrochelatase (FECH) completes heme synthesis by chelating protoporphyrin with iron, but also chelates most of a small remaining amount of protoporphyrin with zinc. As a result, circulating erythrocytes contain small amounts of protoporphyrin, which is mostly zinc chelated rather than metal-free. The presence of zinc protoporphyrin in erythrocytes in health and disease was not known until the 1970s. The term "free erythrocyte protoporphyrin" (FEP) originated before then to describe amounts of what was thought to be metal-free protoporphyrin. Now we know that in almost all of the many conditions that increase erythrocyte protoporphyrin (iron deficiency, lead poisoning, anemia of chronic disease, hemolytic anemias, etc.), the increase is mostly zinc protoporphyrin.
- Metal-free protoporphyrin predominates only in EPP and XLP.
  - EPP is caused by FECH deficiency, so formation of both heme and zinc protoporphyrin is impaired, and the excess protoporphyrin in erythrocytes is mostly metal-free (85-100%). This is a key feature for confirming an EPP diagnosis.
  - XLP is due to ALAS2 gain of function, so amounts of protoporphyrin produced exceed the capacity of normal FECH activity, so both metal-free and zinc protoporphyrin accumulate, with the former usually predominating (50~85%).
- In porphyrias and normal individuals, porphyrin content is highest in young erythrocytes, and decreases as circulating cells age. Therefore, the "fluorocytes" seen on peripheral smears by fluorescent microscopy in EPP are mostly younger erythrocytes.
- Large increases in erythrocyte porphyrins other than zinc protoporphyrin occur only in:
  - CEP, in which uroporphyrin I and coproporphyrin I usually predominate. Protoporphyrin may predominate in mild cases.
  - EPP and XLP, in which metal-free protoporphyrin predominates.

A complete remission of PCT, including normalization of all biochemical abnormalities, can almost always be achieved with adequate treatment. Remission of PCT sometimes results from removal of susceptibility factors, such as cessation of alcohol use. A retrospective diagnosis of PCT is generally impossible by DNA studies, because only ~20% of patients have an inherited heterozygous uroporphyrinogen decarboxylase mutation (18). Symptoms are almost

always life-long in EPP and XLP, so erythrocyte protoporphyrin elevation persists with some variation over time (19).

What medical and laboratory records should patients retain? Patients with porphyria should, at the very least, obtain copies of the biochemical and DNA documentation for their diagnosis and retain them indefinitely. Further testing may be necessary if the diagnosis was not adequately documented.

References cited.

1. Phillips JD, Anderson KE**.** The porphyrias (Chapter 59). In: Kaushansky K, Lichtman MA, Prchal JT, et al., eds. Williams Hematology, 10th edition. New York: McGraw-Hill; 2021:961-86.
2. Whatley SD, Badminton MN**.** Role of genetic testing in the management of patients with inherited porphyria and their families. Ann Clin Biochem. 2013; 50: 204–16.
3. Chen B, Whatley S, Badminton M, Aarsand AK, Anderson KE, Bissell DM, et al. International Porphyria Molecular Diagnostic Collaborative: an evidence-based database of verified pathogenic and benign variants for the porphyrias. Genet Med. 2019;21(11):2605-13.
4. Aarsand AK, Villanger JH, Stole E, Deybach JC, Marsden J, To-Figueras J, et al. European specialist porphyria laboratories: diagnostic strategies, analytical quality, clinical interpretation, and reporting as assessed by an external quality assurance program. Clin Chem. 2011;57(11):1514-23.
5. Sandberg S, Elder GH**.** Diagnosing acute porphyrias. Clin Chem. 2004;50(5):803-5.
6. Woolf J, Marsden JT, Degg T, Whatley S, Reed P, Brazil N, et al. Best practice guidelines on first-line laboratory testing for porphyria. Ann Clin Biochem. 2017;54(2):188-98.
7. Hahn M, Bonkovsky HL**.** Multiple chemical sensitivity syndrome and porphyria. A note of caution and concern. Arch Intern Med. 1997;157:281-5.
8. Thunell S, Harper P, Brock A, Petersen NE**.** Porphyrins, porphyrin metabolism and porphyrias. II. Diagnosis and monitoring in the acute porphyrias. Scand J Clin Lab Invest. 2000;60(7):541-59.
9. Poh-Fitzpatrick MB**.** A plasma porphyrin fluorescence marker for variegate porphyria. Arch Dermatol. 1980;116:543-7.
10. Poh-Fitzpatrick MB, Lamola AA**.** Direct spectrofluorometry of diluted erythrocytes and plasma: a rapid diagnostic method in primary and secondary porphyrinemias. J Lab Clin Med. 1976;87(2):362-70.
11. Longas MO, Poh-Fitzpatrick MB**.** A tightly bound protein-porphyrin complex isolated from the plasma of a patient with variegate porphyria. Clin Chim Acta. 1982;118:219-28.
12. Mauzerall D, Granick S**.** The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J Biol Chem. 1956;219:435-46.
13. Zhang J, Yasuda M, Desnick RJ, Balwani M, Bishop D, Yu C**.** A LC-MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879(24):2389-96.
14. Deacon AC, Peters TJ**.** Identification of acute porphyria: evaluation of a commercial screening test for urinary porphobilinogen. Ann Clin Biochem. 1998;35(Pt 6):726-32.
15. Elder GH, Smith SG, Jane Smyth S**.** Laboratory investigation of the porphyrias. Ann Clin Biochem. 1990;27:395-412.

16. Anderson KE, Bloomer JR, Bonkovsky HL, Kushner JP, Pierach CA, Pimstone NR, et al. Recommendations for the diagnosis and treatment of the acute porphyrias. Ann Intern Med. 2005;142(6):439-50.

17. Gou EW, Balwani M, Bissell DM, Bloomer JR, Bonkovsky HL, Desnick RJ, et al. Pitfalls in Erythrocyte Protoporphyrin Measurement for Diagnosis and Monitoring of Protoporphyrias. Clin Chem. 2015;61(12):1453-6.

18. Jalil S, Grady JJ, Lee C, Anderson KE**.** Associations among behavior-related susceptibility factors in porphyria cutanea tarda. Clin Gastroenterol Hepatol. 2010;8(3):297-302.

19. Gou E WC, Greene T, Anderson KE, Phillips JD**.** A longitudinal analysis of erythrocyte and plasma protoporphyrin levels in patients with protoporphyria. Journal of Applied Laboratory Medicine. 2017;3(2):213-21.